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THE MODE OF ACTION OF THE OXIDASES.[1]

By HERBERT H. BUNZELL.

(*From the Bureau of Plant Industry, United States Department of Agriculture, Washington.*)

(Received for publication, December 14, 1915.)

INTRODUCTION.

This paper deals only with such peculiar conditions existing in fresh plant tissues as are capable of bringing about the oxidation by means of atmospheric oxygen of certain aromatic compounds closely related to phenol, usually provided with two or more OH or CH_3 groups. Inasmuch as these peculiar conditions existing in plants were hitherto and even now are generally explained by assuming the existence of certain chemical compounds of catalytic properties called oxidases, the efficiency of the factors underlying the conditions referred to seems in general correlated with the concentration of the oxidases present in the tissue. Since there is no convincing evidence to the effect that the oxygen transfer mechanism is based purely on the presence of certain compounds, and the reactions exhibited by means of this mechanism may equally well be explained in other ways, the writer has adopted the term "oxidase activity" rather than "concentration of oxidases" to denote the factors responsible for the complex of reactions based on oxygen absorption in the presence of plant juices or tissues. In the writer's mind the concept has for some time existed in the broader form just stated, although in his papers the terms "concentration of oxidases" and "oxidase activity" have been used interchangeably. To express the activity of a plant juice in this respect, the following unit has been accepted provisionally: "An oxidase solution of such strength that 1 liter of it will be capable of bringing about the consumption by the reagent used of the equivalent of 1 gm.

[1] Published by permission of the Secretary of Agriculture.

of hydrogen; *i.e.*, of 8 gm. of oxygen."[2] If instead of a plant juice or a modified plant juice we work with dried plant tissue, the moisture content has to be taken into account in the calculation.

The aromatic compounds that have been used as oxidase reagents are numerous.[3] It has been shown for a number of them, and is presumably true of all, that they undergo a spontaneous oxidation in aqueous solution in the presence of oxygen. The rate of oxidation may be greatly accelerated by means of alkalies, heavy metal ions, and oxidase-active animal or plant tissues or extracts of the same. Such animal or plant tissues or extracts would seem to be, therefore, catalytic agents. In this paper only increased oxygen absorption due to the presence of plant juices or tissues will be discussed.

Numerous experiments embodying the study of the rate of oxidation of some of the oxidase reagents in the presence as well as in the absence of plant juices have been made according to the methods developed in this laboratory.[4] From all these experiments the fact stands out that the accelerating action of the plant material is ended after a definite period. This period ranges within wide limits, according to the experimental conditions, nature of the plant material and reagent used—in our experiments from $\frac{1}{2}$ hour to 3 or 4 hours. To illustrate this point the following experiments were selected at random.

[2] Bunzell, H. H., The Measurement of the Oxidase Content of Plant Juices, *U. S. Dept. of Agriculture, Bureau of Plant Industry, Bull. 238*, 1912, 40.

[3] Kastle, J. H., The Oxidases and Other Oxygen-Catalysts Concerned in the Biological Oxidases, *Bull. Hyg. Lab., U. S. P. H., 59*, 1910. Clark, E. D., The Plant Oxidases, New York, 1910. Begeman, O. H. K., Beiträge zur Kenntnis pflanzlicher Oxydationsfermente, *Arch. f. d. ges. Physiol.*, 1915, clxi, 45.

[4] Bunzell, Ein neuer Apparat zur Bestimmung von Oxydasen in Gewebesäften, *Z. f. biol. Tech. u. Meth.*, 1912, ii, 307; The Measurement of the Oxidase Content of Plant Juices, *U. S. Dept. of Agriculture, Bureau of Plant Industry, Bull. 238*, 1912; A Biochemical Study of the Curly-Top of Sugar Beets, *U. S. Dept. of Agriculture, Bureau of Plant Industry, Bull. 277*, 1913; Quantitative Oxidase Measurements, Orig. Com., *VIIIth Inter. Cong. Applied Chem.*, 1912, xix, 37; Biological Oxidizability and Chemical Constitution, *Jour. Biol. Chem.*, 1914, xvii, p. xxxvi; A Simplified and Inexpensive Oxidase Apparatus, *Jour. Biol. Chem.*, 1914, xvii, 409; Oxidases in Healthy and in Curly-Dwarf Potatoes, *Jour. Agric. Research*, 1914, ii, 373; On Alfalfa Laccase, *Jour. Biol. Chem.*, 1915, xx, 697.

TABLE I.

Material: Dried and Finely Powdered Potato Peels. 0.05 Gm.
Reagent: Pyrogaltol, 0.01 Gm. Small Apparatus. 6 Cc. H_2O.*

Time.	Reading.†
10.30	0
10.45	−1.50
11.00	−1.45
11.15	−1.35
11.30	−1.35
11.45	−1.35
12.00	−1.35

TABLE II.

Material: Dried and Finely Powdered Tulip Tree Buds. 0.01 Gm.
Reagent: Pyrocatechol, 0.01 Gm. Small Apparatus. 6 Cc. H_2O.

Time.	Reading.
10.45	0
11.00	−1.15
11.15	−2.20
11.30	−2.30
11.45	−2.70
1.00	−3.00
1.30	−3.00

* Bunzell, *Jour. Biol. Chem.*, 1914, xvii, 409.
† All readings are expressed in terms of centimeters of mercury.

TABLE III.

Material: Tulip Tree Leaves. 0.10 Gm.
Reagent: Phlorhizin, 0.01 Gm. Small Apparatus. 6 Cc. H_2O.

Time.	Reading.
11.00	0
11.15	−1.40
11.30	−2.25
11.45	−3.00
12.00	−3.40
12.30	−3.80
12.45	−4.00
1.00	−4.00

TABLE IV.

Material: Dried and Finely Powdered Spinach Leaves. 0.10 Gm.
Reagent: p-Cresol, 0.02 Gm. Small Apparatus. 6 Cc. H_2O.

Time.	Reading.
10.15	0
10.30	−0.20
10.45	−0.60
11.00	−0.80
11.15	−1.20
11.30	−1.40
11.45	−1.45
12.00	−1.65
12.15	−1.65
12.30	−1.65

TABLE V.

Material: Dried and Finely Powdered Spinach Roots. 0.025 Gm.
Reagent: Tyrosine, 0.01 Gm. Small Apparatus. 6 Cc. H_2O.

Time.	Reading.
10.00	0
10.45	−1.05
11.00	−1.20
11.30	−1.20
11.45	−1.20
12.30	−1.15
12.45	−1.20

TABLE VI.

Material: Dried and Finely Powdered Canna Stems. 0.02 Gm.
Reagent: Guaiacol, 0.02 Gm. Small Apparatus. 6 Cc. H_2O.

Time.	Reading.
2.15	0
2.30	−1.30
2.45	−1.50
3.00	−1.65
3.15	−1.70
3.30	−1.90
3.45	−1.90

Control experiments with aqueous solutions of the oxidizable materials used in these experiments show no measurable oxygen absorption. This behavior, of course, does not mean that they are not oxidized under the conditions of the control experiments, but that the rate of oxidation is insignificant compared with that taking place in the presence of plant materials.

There are a number of causes which might account for the rather abrupt ending of the influence of the plant material. The writer set out to determine whether the standstill which is apparent in all the oxidase reactions studied is due to the exhaustion of the agents responsible for the oxidase activity or to some inhibitory influence arising in the course of the reaction. The following inhibitory factors were considered.

1. For reasons of technique the experiments were mostly carried out at 37°C., a temperature which may be somewhat destructive to the agencies involved.

2. Shaking is known to have a deleterious influence on some enzymes and may also affect the agencies involved.

3. The reagents used may have an inhibiting effect on the reaction.

4. The products formed in the course of the oxidation may have a retarding and even an inhibiting influence on the reaction, by driving the reaction in the opposite direction, by combining with the active substances present, or by unfavorably changing the reaction of the medium.

5. The available oxidizable material may be used up.

6. The available oxygen may be consumed or its diminished pressure may have a retarding effect.

Experiments were therefore planned to determine the influence of these factors.

EXPERIMENTAL.

1. Effect of Different Temperatures on the Measure of Oxidase Activity.

The results in Table VII indicate that during shaking at the higher temperatures a part of the oxidase activity is destroyed. In general there is a marked diminution in activity as the temperature rises to 47°C.; below that the effect is not so marked

TABLE VII.

Influence of Different Temperatures on Oxygen Absorption. Rate of Shaking: 5 Excursions in 3.2 Seconds. Material: Finely Powdered Dried Potato Peels. In the Case of Solids, 0.01 Gm. Was Used; in the Case of Liquids, Two Drops.

	Pyrogallol.	Pyrocatechol.	Hydroquinone.	Phlorhizin.	Guaiacol.	Tyrosine.	*m*-Cresol.	*p*-Cresol.	Eugenol.	Isoeugenol.
	Gm. of material used.									
	0.05	0.02	0.02	0.02	0.05	0.10	0.05	0.01	0.10	0.10
°C.	Absorption.									
26.5	1.10	1.25	2.65	1.60	1.00	1.30	2.20	4.40	0	0.20
31.0	1.40	1.35	2.50	2.00	1.70	1.60	2.10	Leak	0.20	0.60
37.5	0.90	0.90	1.25	1.20	1.70	1.40	2.50	3.40	0.10	0.40
47.4	1.05	0.90	0.90	0.90	1.50	1.05	1.60	Leak	0.20	0.80

and might well be only apparent, due to the experimental fluctuations. On account of these fluctuations it did not seem necessary to reduce the manometer readings to a uniform temperature. The temperature correction would amount to only 3 per cent for a difference in temperature of 10°C., or less than 1 mm. in an absorption corresponding to 3 cm. of mercury.

Even at a temperature as high as 47.4°C. there is still considerable activity. The rather high temperature, therefore, (37°C.) at which most of the work so far has been carried out, probably has a retarding but not an inhibiting effect on the oxidase activity of the plant tissues used in this work.

2. Effect of the Rate of Agitation on the Measure of Oxidase Activity.

From Table VIII it is apparent that the rate of shaking within the limits of these experiments has no marked influence on the final results. The important effects of agitation, namely, the saturation of the solution with oxygen from the air and the distribution of the active plant tissue particles, can be accomplished by shaking with 15 excursions per minute just as well as with 150 excursions per minute.

TABLE VIII.

Influence of the Rate of Agitation on the Oxygen Absorption. Temperature: 37.2°C. Material: Finely Powdered Dried Potato Peels. In the Case of Solids, 0.01 Gm. Was Used; in the Case of Liquids, Two Drops.*

	Pyrogallol.	Pyro-catechol.	Hydro-quinone.	Phlorhizin.	Guaiacol.	Tyrosine.	*m*-Cresol.	*p*-Cresol.	Eugenol.	Iso-eugenol.
Gm. of material used.....	0.05	0.02	0.02	0.02	0.05	0.10	0.05	0.01	0.10	0.10
5 excursions in 2°C.....	1.20	1.30	2.40	1.70	1.30	1.60	2.10	3.75	0.05	0.10
5 " " 3.2°C.....	1.10	1.25	2.65	1.60	1.00	1.30	2.20	4.40	0	0.20
5 " " 5.4°C.....	1.00	1.20	2.20	1.40	1.00	1.75	2.25	4.00	0	0
15 " " 10 sec. once every min.....	1.10	1.30	1.70	1.80	1.20	1.60	2.30	3.40	0.20	0.60

* In this connection the results of Appleman are interesting. Using pyrogallol as reagent and potato juice as plant material, he found that by mild agitation at long intervals the oxygen absorption can be made to spread over several days. (Appleman, C. O., *Maryland Agricultural Experiment Station, Bull. 183*, 1914, 193.)

3. Inhibiting Effect of the Reagents Used on the Measure of Oxidase Activity.

In order to determine what effect is produced on the end-results by an excess of the reagents, it was necessary first to establish what constitutes such an excess. Table IX presents results which were obtained by working with fixed quantities of plant material and varying quantities of reagents.

These results show that *p*-cresol when used in quantities of more than three drops (0.036 gm.) in 6 cc. of liquid retarded the action. There also seems to be a slight retardation in the case of pyrogallol when more than 0.015 gm. was used. For the particular sample of potato material used, 0.01 to 0.015 gm. of the solid reagents seems to be a desirable quantity to use; in the case of the three liquids the situation is less simple; to obtain the maximum result, *p*-cresol would seem to be used best in quantities of two drops (0.024 gm.); *m*-cresol, three to four drops (0.036 to 0.048 gm.); and guaiacol in quantities of four drops (0.056 gm.). These ratios, however, would probably vary according to the particular plant material used and the working conditions.

There seems to be, therefore, no marked retardation due to most of the reagents present, even if they are used in considerable excess.

TABLE IX.

Effect on the End-Readings of the Reagents Used. Temperature: 37°C. Material: Finely Powdered Dried Potato Peels. The Quantity of Material Used in Each Case is Given in the First Perpendicular Column with the Name of the Reagent.

Gm. of reagent.	0.02	0.02	0.015	0.015	0.01	0.01	0.005	0.005	0.0025	0.0025
Pyrocatechol. 0.02 gm. potato powder	1.35	1.50	1.40	1.30	1.20	1.30	1.20	1.10	1.00	Lost.
Phlorhizin. 0.02 gm. potato powder	1.60	2.20	2.00	Lost	1.40	1.60	0.80	1.00	0.20	0.20
Hydroquinone. 0.02 gm. potato powder	2.15	2.00	2.00	2.30	2.00	2.10	1.90	2.10	1.60	1.60
Pyrogallol. 0.05 gm. potato powder	1.15	1.25	1.40	1.50	1.25	1.50	1.60	1.50	1.05	1.00
Tyrosine. 0.10 gm. potato powder	1.40	1.40	1.50	1.50	1.20	1.25	1.05	1.35	0.80	1.05
Quantity of reagent (drops).	5*	5	4	4	3	3	2	2	1	1
Guaiacol. 0.05 gm. potato powder	1.90	2.05	1.90	1.90	1.25	1.40	0.90	1.00	0.50	0.45
m-Cresol. 0.05 gm. potato powder	2.60	2.40	2.55	2.30	2.00	2.00	1.60	1.80	1.10	1.10
p-Cresol. 0.01 gm. potato powder	2.60	2.40	3.00	3.70	3.70	3.90	3.85	3.80	3.30	3.30

* The pipette used for all the experiments was calibrated and it was found that each drop of guaiacol weighed 0.014 gm.; each drop of *m*- and of *p*-cresol, 0.012 gm.

4. Effect of Products Formed in the Course of the Reaction on the Measure of Oxidase Activity.

In order to determine whether the products formed are responsible for the cessation of oxygen absorption, the reaction was allowed to come to an end as usual with the ten reagents used and

then once more the same quantities of the plant material were added as in the beginning, and the shaking was continued. The final results are given in Table X.

TABLE X.

Influence of the Products Formed in the Course of the Reaction on the Oxygen Absorption. Temperature: Room. Material: Finely Powdered Dried Potato Peels. In the Case of Solids, 0.01 Gm. Was Used; in the Case of Liquids, Two Drops.

	Pyrogallol.	Pyrocatechol.	Hydroquinone.	Phlorhizin.	Guaiacol.	Tyrosine.	*m*-Cresol.	*p*-Cresol.	Eugenol.	Isoeugenol.
Gm. of material used.....	0.05	0.02	0.02	0.02	0.05	0.10	0.05	0.01	0.10	0.10
First set of results.....	1.40	1.35	2.50	2.00	1.70	1.60	2.10	Leak	0.20	0.60
After addition of more powder in the same quantities as before.....	1.40	0.45	1.40	1.75	3.60	3.05	4.30	2.00	0.10	0.50

It is evident that the activity of the plant powder is not paralyzed by the products formed in the course of the reaction. While in the case of some reagents the final result is somewhat reduced, it is augmented in others, unaffected in a few, but completely inhibited in none.

5. Consumption of the Available Oxidizable Material.

Table X gives evidence that the complete utilization of the oxidizable material is not the reason for the cessation of the reaction. As the second row of figures shows, absorption goes on after the addition of fresh plant material without the addition of further quantities of reagents.

6. Consumption of Available Oxygen and the Retarding Effect on the Results of the Diminished Partial Pressure of the Oxygen Present.

It seems improbable that in any one experiment the total available quantity of oxygen should be consumed. If we consider one-fifth of the gas in the apparatus as oxygen and assume

that the change of pressure is entirely due to absorption of oxygen, the extreme variation of pressure as registered by the attached manometer, *i.e.*, 8 cm., would represent the absorption of only about one-half of the oxygen present. If, however, gases are produced in the process, the same change in pressure would correspond to a greater oxygen absorption. To prove conclusively that the consumption of oxygen is not the limiting factor, several series of experiments were carried out with varying quantities of the plant material and different reagents.

TABLE XI.

Results Obtained with Varying Quantities of Plant Material. Temperature: 37°C. Material: Finely Powdered Dried Potato Peels.

Reagent.											
Pyrocatechol 0.02 gm.	Gm. of material used	0.01	0.01	0.02	0.02	0.03	0.03	0.04	0.04	0.05	0.05
	Reading	0.65	0.65	1.20	1.30	1.80	1.90	2.70	2.80	3.00	3.10
Pyrogallol 0.015 gm.	Gm. of material used	0.02	0.02	0.04	0.04	0.06	0.06	0.08	0.08	0.10	0.10
	Reading	0.10	0.10	0.50	0.50	1.00	1.00	1.80	1.80	2.00	2.00
Hydroquinone 0.015 gm.	Gm. of material used	0.01	0.01	0.02	0.02	0.03	0.03	0.04	0.04	0.05	0.05
	Reading	1.00	1.10	0.40	1.90	3.80	3.20	4.70	4.50	5.50	5.90
Phlorhizin 0.02 gm.	Gm. of material used	0.01	0.01	0.02	0.02	0.03	0.03	0.04	0.04	0.05	0.05
	Reading	1.00	1.20	2.00	1.90	2.60	3.10	3.40	3.90	4.20	4.00
Tyrosine 0.015 gm.	Gm. of material used	0.05	0.05	0.10	0.10	0.15	0.15	0.20	0.20	0.25	0.25
	Reading	0.50	0.60	1.40	1.15	1.90	2.00	2.50	2.70	3.00	3.00

DISCUSSION.

As these figures show, the final results for each reagent are practically proportional to the quantity of active material used. The deviations from an absolute and direct proportionality lie within the limits of error of experimentation. The definite termination of the reactions when comparatively small quantities of oxidase material were used is not due, therefore, to the con-

sumption of the oxygen present or to its diminished partial pressure, but to complete utilization of the plant material.

The termination of the oxidation of the artificial chromogens under discussion, therefore, is due to the exhaustion of the activity of the plant material. If we wish to consider the activity of the plant juices or plant tissues responsible for the oxygen transfer or catalytic action, we may say that the compounds or the mechanism responsible for the process have become destroyed or injured and have thereby lost their power to act catalytically.

The writer does not contend that the factors mentioned and discussed in the foregoing pages are without influence on the end-result. In many instances they seem to have a decided influence, usually retarding, though sometimes stimulating, but they are not either individually or collectively the cause for the sharp end-point obtained in the course of the oxygen absorption. Even if the factors stated were responsible for the existence of an end-point, we should be justified, for lack of better methods, in using as indicator of the oxidase activity the figures obtained by means of the measurement of the oxygen absorption, because the figures obtained would still be comparable. The greater the original activity, the greater we should expect the final result to be, for this would be a result of the original activity and a number of destructive agencies which would be fairly uniform in all cases.

There is also to be considered the presence of natural chromogens in the material used, which in the presence of the oxidizing enzymes would absorb an appreciable quantity of oxygen. That such natural chromogens are widely existent in many kinds of plant materials, and perhaps in all, is well known. As a concrete example it may be stated that the chromogens in 0.10 gm. of finely powdered tulip tree buds (dried *in vacuo*) brought about in the small apparatus[5] a diminution of pressure of 0.50 to 0.80 cm. These chromogens seem to be found, to some extent at least, in the juice pressed out of the leaves. In the case of tomatoes, with which many experiments were tried, 2 cc. of leaf juice brought about in the small apparatus a diminution of pressure of 1.10 to 1.60 cm. of mercury. Tobacco leaf juice caused a reduction of

[5] Bunzell, A Simplified and Inexpensive Oxidase Apparatus, *Jour. Biol. Chem.*, 1914, xvii, 409.

pressure of 1.75 cm. of mercury. The chromogens, in the case of the tulip tree buds at least, are oxidized by the aid of oxidases. After the powder was boiled in water for 1 minute it was deprived of the power to oxidize either the reagents here used or the natural chromogens. Dried leaf powder of the tulip tree seems to have a chromogen content of about twice that of the buds, as evidenced by the oxygen absorption.

When both oxidase reagent and chromogen are present, with excessive oxidase activity and oxygen, the result (in the case of tomato leaves) is by no means a simple summation of the oxygen absorption of the two. The oxidase activity seems to divide itself between the artificial and the natural chromogens, presumably entering into some preliminary stage with both of them. In this way combination with the artificial chromogen seems to take place, whether the latter is markedly oxidized or not. Presence of a scarcely oxidizable chromogen may in this way greatly depress the oxidation of a natural chromogen. The presence of the reagent pyrocatechol, for example, seems to prevent the oxidation of the chromogen. This phenomenon is illustrated by the following experiment. In parallel experiments diluted tomato juice alone, as well as the same plus pyrocatechol, were shaken and the oxygen absorption was determined in the usual way. The details of the experiment are described in Table XII.

TABLE XII.

Oxidation of Chromogen in Tomato Juice + Oxidation of Chromogen + Oxidase Reagent (Pyrogallol) Combined. Temperature: 37.2°C. Rate of Shaking: 5 Excursions in 3.4 Seconds.*

Time.	0.01 gm. pyrocatechol + 4 cc. 50 per cent tomato juice + 2 cc. H_2O.	0.01 gm. pyrocatechol + 4 cc. 50 per cent tomato juice + 2 cc. H_2O.	4 cc. 50 per cent tomato juice + 2 cc. H_2O.
11.15	0	0	0
11.30	0.10	0.10	0.55
11.45	0.20	0.20	0.60
12.00	0.35	0.30	0.90
12.15	0.20	0.20	0.75
12.30	0.15	0.20	0.75

* These results were copied from data secured in the course of a cooperative work with Professor R. W. Thatcher, of the University of Minnesota, and are published here with his permission.

The example above shows that we would not be justified in correcting our results obtained for the oxidation of pyrocatechol in the presence of tomato juice by mere subtraction of the figures obtained from the absorption of oxygen by the corresponding amount of tomato juice alone. The distribution of the chromogens is being studied in the laboratory at present and later a general paper will appear discussing their occurrence in plants and their significance.

We may conclude, therefore, that the figures which were secured throughout this work are the result of a number of factors, some having an augmenting effect on the end-result, others a depressing effect. There are undoubtedly other factors which come into play. The presence of reductases[6] and the concentration of hydrogen ions[7] undoubtedly have an influence on the result, but the nature of their action is too obscure as yet to allow any definite statements concerning them. While the question of reductases is far from being elucidated on account of lack of proper methods, the influence of the reaction of the medium is susceptible to accurate experimentation. The writer is engaged with the latter at present.

Although we have to recognize the retarding influence of the various factors mentioned, it is also evident that none of them are responsible for the prompt standstill invariably shown by the oxygen absorption. This phenomenon is peculiar to the agencies responsible for the oxygen absorption. If chemical compounds, the so called oxidases, are responsible for the absorption, then the oxidases become used up in the course of the reaction; if the peculiar condition of the colloids present accounts for the reaction, then a rearrangement into an inactive condition takes place during the reaction.

[6] Reed, G. B., Evidence for the General Distribution of Oxidases in Plants, *Bot. Gaz.*, 1915, lix, 407.

[7] Rose, D. H., Oxidation in Healthy and Diseased Apple Bark, *Bot. Gaz.*, 1915, lx, 55. (Also indicated by some preliminary experiments of the writer.)

THE RELATIVE OXIDASE ACTIVITY OF DIFFERENT ORGANS OF THE SAME PLANT.[1]

By HERBERT H. BUNZELL.

(From the Bureau of Plant Industry, United States Department of Agriculture, Washington.)

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At the meeting of the American Society of Biological Chemists in 1913, a paper[2] was presented by the writer on the correlation of biological oxidizability and chemical constitution. By biological oxidizability is meant the ease with which certain aromatic compounds are oxidized by means of atmospheric oxygen in the presence of oxidase-active plant material.

The results given represent experiments carried out with the following eighteen reagents: pyrocatechol, resorcinol, hydroquinone, guaiacol, pyrogallol, phloroglucinol, α-naphthol, leuko-base of malachite green, aloin, benzidine, tyrosine, phlorhizin, *o*-cresol, *m*-cresol, *p*-cresol, *o*-toluidine, *m*-toluidine, and *p*-toluidine. Juices of shoots, tubers, and leaves of healthy potato plants and of plants having the curly-dwarf disease were the oxidase-active materials used. For convenience of comparison the results were presented graphically, the oxidase activities being laid off on the ordinates, and the organic compounds marked at equal intervals on the abscissæ. By connecting the points a line is obtained which gives a composite picture of the activity of the particular plant material. Six lines were obtained in this way, each representing the oxidizing power of a different type of potato material.

To quote from the paper referred to above[2]: "There is one striking feature about these six curves: They all show a similar trend. The elevations and depressions of each curve follow very closely those of the other curves. Let us take it for granted that the relationships found in the juice are indicative of conditions in the plant cell. Then we might say that the conditions in the cells which control the oxidizing powers toward these eighteen reagents are about the same in all parts of the potato plant. It may be that other plants show the same oxidase relationships or that each plant has its own type of curve representing its oxidase activities. The latter situation would be particularly interesting. We would gain access to some of the metabolic functions characteristic of the particular plant in question. Such specific enzyme relationships in animals have been dis-

[1] Published by permission of the Secretary of Agriculture.

[2] Bunzell, H. H., *Jour. Biol. Chem.*, 1914, xvii, p. xxxvi.

covered in the case of the enzymes involved in nuclein metabolism (Walter Jones)."

Since the paper mentioned above was read, a number of other plants have been studied with respect to their oxidase activities. In this connection the potato, onion, tulip tree, spinach, and sugar beet have been studied. The experiments were carried out with the following organs: potato leaves, potato tubers, onion leaves, onion bulbs, tulip tree leaves, tulip tree buds, spinach leaves, and spinach roots. All results represent the mean of a number of experiments carried out on two or more samples.

The data for the potato are taken from a previous publication.[3] Only the figures representing the normal material were used. In the case of the onion (red danvers), the shoots and bulbs of the young plants (about 4 inches above ground) were dried in a desiccator over lime, powdered, and examined in the usual way. It turned out that neither bulbs nor foliage had any activity toward any of the reagents tried. The results obtained with the tulip tree material were taken from data obtained in cooperative work with Dr. William Crocker of the University of Chicago, at present Plant Physiologist in the Bureau of Plant Industry. The experiments were carried out with dried and powdered material, and the data were calculated on the basis of the original moisture content. In the case of the buds the data represent three collections made at different times of the year from five different trees, and two or three determinations with each reagent for each sample collected. Table I gives the average results.

TABLE I.

*Mean Results Obtained in the Study of the Oxidase Activity of Tulip Tree Buds. The Results Are Expressed in Terms of Units.**

Reagents.	Pyrogallol.	Pyrocatechol.	Hydroquinone.	Phlorhizin	Guaiacol.	Tyrosine.	*m*-Cresol.	*p*-Cresol.	Eugenol.	Iso-eugenol.
Buds two pairs of scales off.	1.22	8.48	6.13	0.44	0.48	0.20	0.30	0.64	0.23	0.23
Inner pair of scales.....	1.15	6.70	3.04	0.40	0.39	0.17	0.29	1.04	0.18	0.21
Leaves.....	0.68	2.23	1.59	0.58	0.48	0.29	0.39	1.58	0	0

* "A unit is an oxidase solution of such strength that 1 liter of it will be capable of bringing about the consumption by pyrogallol of the equivalent of 1 gm. of hydrogen; *i.e.*, the consumption of 8 gm. of oxygen." (Bunzell, The Measurement of the Oxidase Content of Plant Juices, *U. S. Dept. of Agriculture, Bureau of Plant Industry, Bull. 238*, 1912, 40.)

[3] Bunzell, *Jour. Agric. Research*, 1914, ii, 373.

The results with spinach represent the mean of duplicates and triplicates carried out in three collections, roots and leaves (freed from midribs) separately (Table II).

TABLE II.

Results Obtained in the Study of Oxidase Activities of Spinach Plants. The Results Are Expressed in Terms of Units.

Reagents.	Pyrogallol.	Pyrocatechol.	Hydroquinone.	Phlorhizin.	Guaiacol.	Tyrosine.	*m*-Cresol.	*p*-Cresol.	Eugenol.	Iso-eugenol.
Leaves...	0.080	0.242	0.023	0	0	0	0	0.084	0	0
Roots ...	0.166	0.312	0.590	0.274	0.197	0.562	0.300	2.434	0.003	0

In the case of sugar beets (young greenhouse plants) the data are less complete; reliable data for only six reagents are at hand (Table III). They are the following: pyrogallol, pyrocatechol, hydroquinone, phlorhizin, guaiacol, and tyrosine.

TABLE III.

Results Obtained in the Study of Oxidase Activities of Sugar Beets. The Results Are Expressed in Terms of Units.

Reagents.	Pyrogallol.	Pyrocatechol.	Hydroquinone.	Phlorhizin.	Guaiacol.	Tyrosine.
Roots.....	0.212	0.330	0.181	0.062	0.259	0.448
Leaves.....	0.196	0.273	0.196	0.080	0.234	0.354

In correlating the various data, it seemed interesting to examine the results relating to the same plant from the following points of view: (1) How does the relative oxidase activity exhibited by any plant material toward the reagents tried compare with the relative oxidase activity exhibited by other portions of the same plant toward the same reagents? (2) How does the general oxidase activity of certain plant organs compare with plant organs with fundamentally different functions?

To facilitate comparison of the data, it was necessary to record them in some graphic form. The circle was therefore divided into ten equal sections. Each diameter was made to represent a reagent and the activities of the plant materials toward the various reagents were laid off on the corresponding diameters.

By connecting the points obtained in this way a graph was secured which represents the oxidase activity of the plant material studied. By recording separately the activities of different parts of the same plant on the same diagram, it is possible to make immediate comparison. Diagrams are presented for the five plants studied.

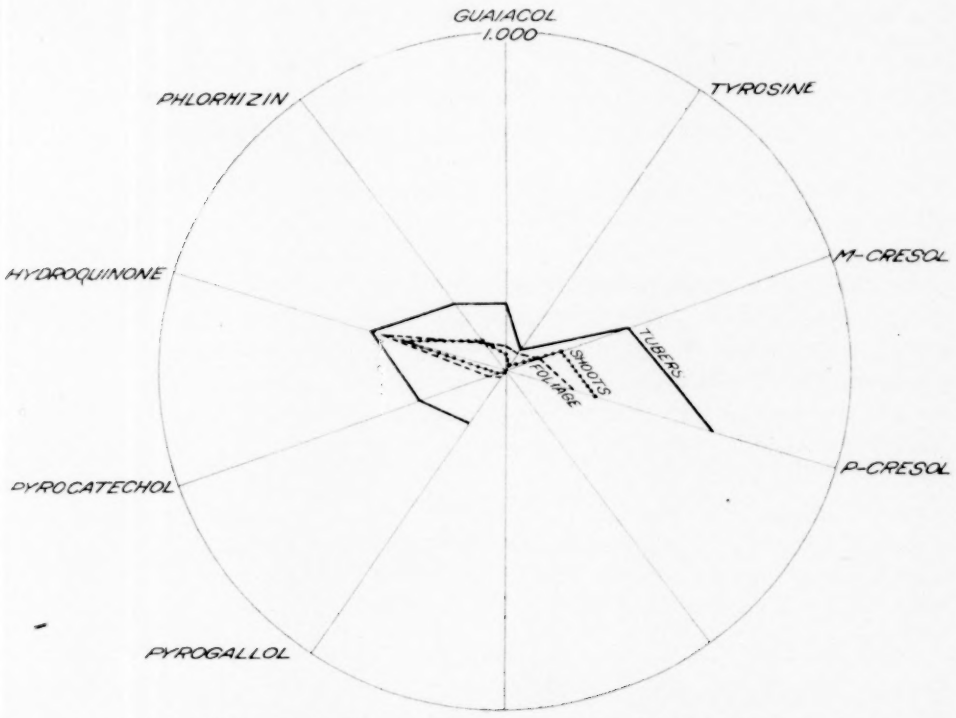

FIG. 1. Oxidase activities of different parts of the potato plant.

As can readily be seen there is a marked parallelism between the graphs representing the oxidase activities of different parts of the potato, tulip tree, and sugar beet. In the case of the onion there is no activity shown by either leaves or roots. With spinach the two graphs are entirely at variance. This non-parallelism between the oxidase activities of spinach roots and spinach leaves, however, may be only apparent. The activity of the leaves is very slight and may become obscured by the presence of chromogens, as pointed out in the previous paper.[4]

The diagrams show that the oxidase situation in the case of spinach is entirely different from the oxidase situation in the case

[4] Bunzell, *Jour. Biol. Chem.*, 1916, xxiv, 91.

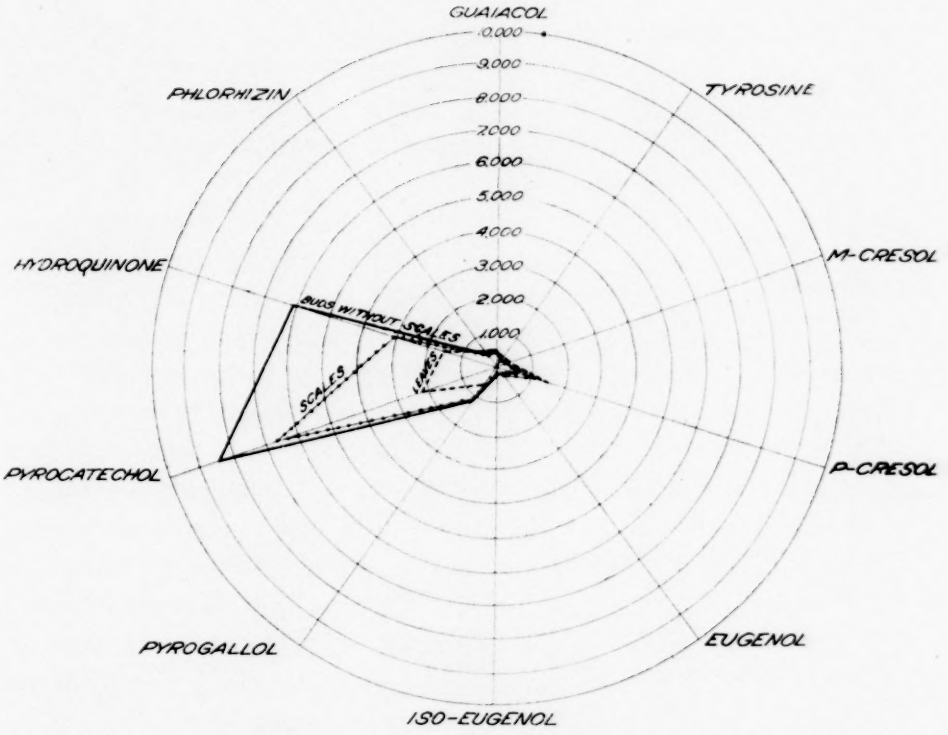

FIG. 2. Oxidase activities of different parts of the tulip tree.

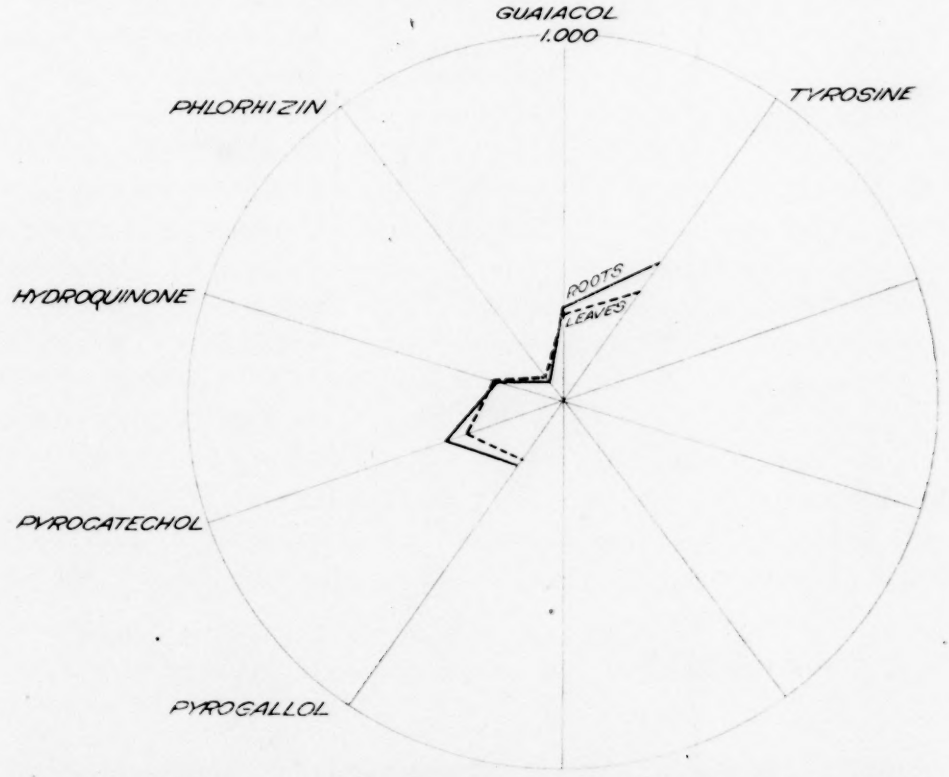

FIG. 3. Oxidase activities of different parts of the sugar beet plant.

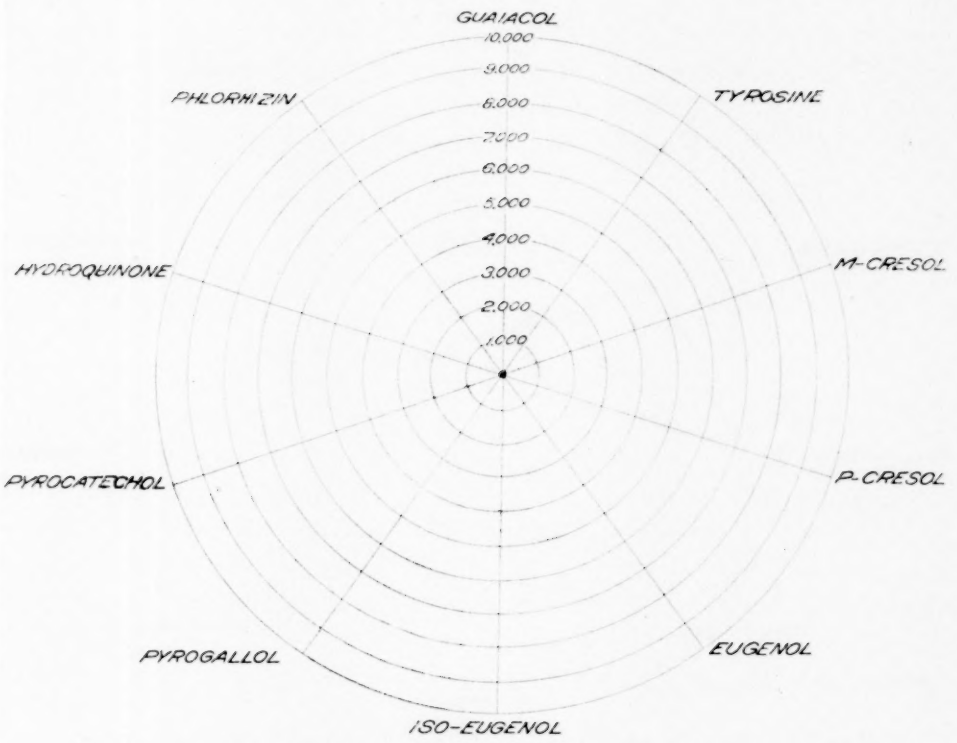

FIG. 4. Oxidase activities of different parts of the onion plant.

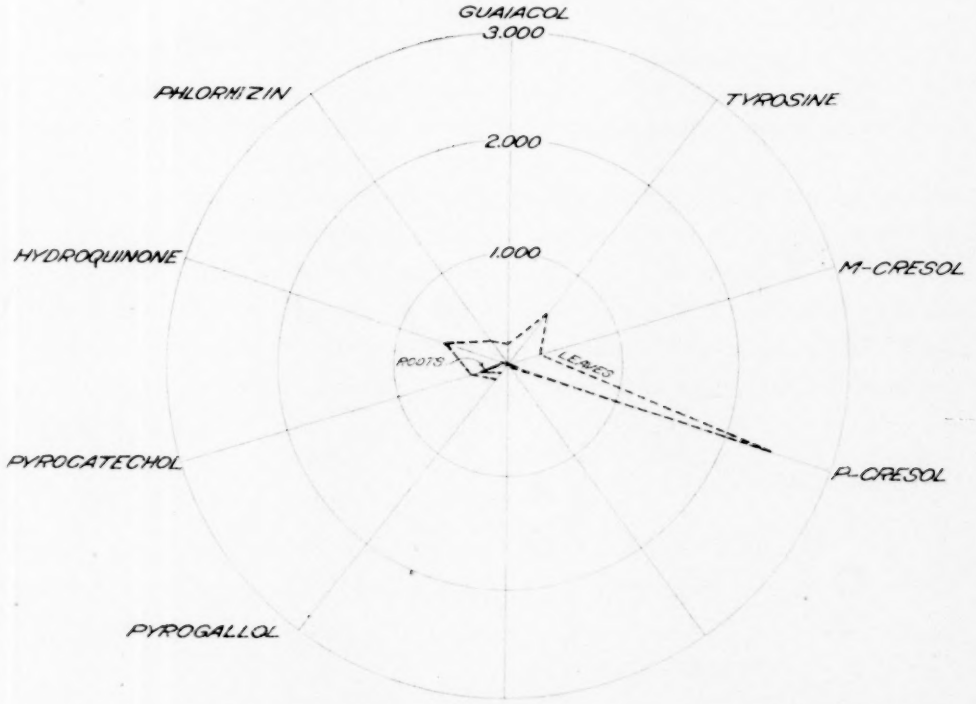

FIG. 5. Oxidase activities of different parts of the spinach plant.

of sugar beet, which in turn is different from that in the case of potato, tulip tree, or onion. The relative oxidase activity as manifested toward the reagents tried is approximately the same for the different parts of the same plant, but different for the different plants tried. It appears then that the second of the two assumptions made above, *viz.*, that each plant has its characteristic relationships, is correct.

What does this mean? To answer this question it would be necessary to know and to take account of all the factors entering into the complex of reactions summarized as oxidase activity. A number of these factors have been pointed out in the previous paper.[4] The fact that in general there is a well marked parallelism existing between the graphs of different organs of the same plant, would indicate that one of these factors must predominate strongly above the others. Our knowledge at present allows us only to surmise what this factor may be. If we assume that the oxidase activity of the plant juices is due to the presence of specific oxidases, as Bach and Chodat claim, then it will be difficult to understand why the oxidation of each reagent should be equally lower in some parts of a plant as compared with other parts of the same plant. If, on the other hand, we assume that the oxidations in the presence of the plant material are carried out principally by virtue of the physicochemical conditions created by the addition of the plant material, an explanation seems nearer at hand. In fact, Traube[5] in his paper on catalysis recognized surface tension as one of the important factors in the acceleration of biochemical reactions. The oxidase activities observed might simply be due to an increased concentration of the oxidizable material or of oxygen, or of both in the layers adjacent to certain colloidal particles. A similar view has been expressed by H. E. Armstrong and coworkers.[6] The particular nature of the colloidal particles present might be a determining factor for the relative concentration of the various oxidizable substances, and thereby for the relative extent of oxidation of these reagents. The actual concentration of the colloids, on the other hand, might be responsible for the general intensity of the oxidase activity. Taking it for

[5] Traube, J., *Arch. f. d. ges. Physiol.*, 1913, cliii, 309.

[6] Armstrong, H. E., Benjamin, M. S., and Horton, E., *Proc. Roy. Soc., Series B*, 1912–13, lxxxvi, 328.

granted then that a certain group of colloids of this nature is being developed by the cells of a certain plant, it would be easy to see why cells in certain organs, generally more active, should produce more of such colloids than others generally less active.

The capacity of certain organs to manufacture colloids of a highly specific nature is illustrated by the work of Reichert.[7] He carried out extensive investigations on starches of different genera, species, varieties, and hybrids, as well as on starches obtained from different parts of the same plant. He studied the appearance of the starch grain, the color reactions, and certain physical constants of the starches, and concluded that every genus and species has a form of starch which is specific and constant in relation to that genus or species. It is probable that similar relationships will be found in the characteristics discussed in this paper. If the oxidase activities observed are due to the presence of certain colloids, this situation is probable.

There remains another aspect of the situation to be discussed. In the metabolism of the plant there are innumerable compounds which are being oxidized in the cells, presumably with the aid of oxidase action. Some of these compounds are probably related to the various aromatic compounds mentioned in this paper. In the light of the findings stated in this paper it would seem that the capacity of the plant to oxidize these various compounds would be characteristic of the particular plant, but would vary in intensity, and in intensity only, in the different parts of the same plant. This line of reasoning would furnish us with direct proof of the existence of a distinct and characteristic type of metabolism for each plant. It remains to be seen to what extent plants of the same genus or species resemble each other in their metabolism.

[7] Reichert, E. T., The Differentiation and Specificity of Starches in Relation to Genera, Species, etc., *Carnegie Institution of Washington, Publication No. 173*, 1913. Also *Science*, 1914, xl, 649.

CEPHALIN.[1]

III. CEPHALIN OF THE EGG YOLK, KIDNEY, AND LIVER.

PRELIMINARY PAPER.

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(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, December 31, 1915.)

The principal object of the present investigation was the search for a convenient source of cephalin. It is true that the brain tissue contains a high proportion of this substance, but it also contains a variety of other phosphatides. Experience has shown that because of this the purification of cephalin from the brain tissue met with great obstacles. It was hoped that in some other organ the distribution of phosphatides might be so much in favor of cephalin that it could be readily freed from impurities. Unfortunately this expectation was not realized for the present. The brain still remains the most convenient source of cephalin. On the other hand, the results of the present work have shown that the cephalins obtained from various organs and from egg yolk possess identical properties and composition, and hence the results of the structural analysis of one will apply to all.

The following table contains the results of the ultimate analysis of cephalin prepared by different workers.

Author.	Source.	C	H	N	P
Levene and West[1]	Brain	60.49	9.52	1.96	3.80
Stern and Thierfelder[2]	Egg yolk	59.68	9.74	1.57	3.64
Levene and West	"	60.00	9.62	1.78	3 69
Frank[3]	Liver	57.10	9.62	1.72	3.91
Levene and West	"	60.33	9.01	1.86	3.75
" " "	Kidney	60.17	8.95	1.70	3.65

[1] Levene, P. A., and West, C. J., *Jour. Biol. Chem.*, 1913–14, xvi, 419; 1916, xxiv, 41.

[2] Stern, M., and Thierfelder, H., *Z. f. physiol. Chem.*, 1907, liii, 379.

[3] Frank, A., *Biochem. Z.*, 1913, l, 277.

From the table it is seen that from egg yolk the material obtained by us did not differ from that prepared by Stern and Thierfelder, although we employed a more rigid procedure of purification. On the other hand, the substance isolated by us from the liver was undoubtedly in a higher state of purity than that described by Frank, and finally in regard to the kidney the present is the first report of the isolation of cephalin from that organ.

Work on other organs is in progress.

EXPERIMENTAL PART.

Egg Yolk Cephalin.

Earlier workers and many of the later workers in lipoid chemistry considered all of the lipoid fraction of egg yolk as lecithin.[4] Erlandsen[5] was the first to question the composition of the lecithin fraction. During the course of his work on the lipoids of heart muscle, he attempted to isolate cuorin from egg yolk, but was unsuccessful. In the same year Stern and Thierfelder[2] reported the isolation of an alcohol-insoluble fraction from the so called lecithin. The compound obtained from fresh eggs resembled cephalin in its properties, and analyzed for the theoretical cephalin composed of stearic and cephalinic acids, glycerophosphoric acid, and amino-ethyl alcohol.[6] The cephalin from a commercial egg preparation, however, showed the composition found by other authors; namely, C, 59.68; H, 9.74; N, 1.57; P, 3.64. The only explanation offered for the different results was that possibly the age of the egg had some effect. We have repeated the work on the egg yolk, using commercial egg yolk powder.

Alcohol Extraction.

Egg yolk powder was extracted four or five times with hot alcohol, the alcoholic extract concentrated and cooled at 0°.

[4] For example, Serono, C., and Palozzi, A. (*Arch. farm. sperim.*, 1911, xi, 553), state that 11–12 per cent of the egg yolk is lecithin, but mention no other lipoid. It is interesting in this connection to compare the work of Barbieri, N. A. (*Compt. rend. Acad. d. sc.*, 1910, cli, 405), who denies the existence of free or bound lecithin in the egg yolk.

[5] Erlandsen, A., *Z. f. physiol. Chem.*, 1907, li, 150.

[6] See Levene and West, *Jour. Biol. Chem.*, 1916, xxiv, 41.

The egg oil and lipoids separated as a semisolid mass. Since it was impossible to filter this, even at 0°, the entire product was stirred with a large quantity of dry acetone. This dissolved the egg oil and considerable quantities of lipoid. The acetone extract was concentrated and again extracted with dry acetone, and the process repeated until the oil dissolved completely in the acetone. All the lipoid fractions were then extracted with ether, and the small amount of sphingomyelin was removed by filtration or centrifugation. The ether-soluble fraction was then separated by precipitation with absolute alcohol into the cephalin (alcohol-insoluble) and lecithin (alcohol-soluble) fractions.

This crude cephalin fraction contained 1.8 per cent nitrogen. When treated with alcohol at 60–70°, as recommended by MacLean for the preparation of cuorin, a product was obtained with the same composition:

0.500 gm. substance neutralized 6.18 cc. 0.1 N acid.
0.300 " " gave 0.0390 gm. $Mg_2P_2O_7$.

	N	P
Found	1.72	3.62

The material was then precipitated from alcohol at 60°, in the same manner as described for the purification of brain cephalin. The two fractions (I, insoluble, and II, soluble) showed the same composition:

I. 0.500 gm. substance neutralized 6.34 cc. 0.1 N acid.
0.300 " " gave 0.0386 gm. $Mg_2P_2O_7$.
II. 0.500 " " neutralized 6.48 cc. 0.1 N acid.
0.300 " " gave 0.0404 gm. $Mg_2P_2O_7$.

Found:	N	P
I	1.75	3.60
II	1.81	3.74

Finally, the ether solution was shaken with dilute hydrochloric acid and the resulting solution repeatedly precipitated with acetone.

0.500 gm. substance neutralized 6.33 cc. 0.1 N acid.
0.300 " " gave 0.0398 gm. $Mg_2P_2O_7$.
0.1484 " " " 0.3265 " CO_2 and 0.1276 gm. H_2O.
0.300 " " " 0.0662 " AgI (glycerol determination).

	C	H	N	P	Glycerol.
Found	60.00	9.62	1.78	3.69	8.65

Ether Extraction.

In order to determine whether the initial solvent used had any effect upon the composition of the alcohol-insoluble fraction, a second lot of egg powder was extracted repeatedly with ether at room temperature. The combined ether extract was treated as given above. The fraction, insoluble in alcohol at 60°, had the following composition:

0.500 gm. substance neutralized 6.53 cc. 0.1 N acid.
0.300 " " gave 0.0400 gm. $Mg_2P_2O_7$.
0.1516 " " " 0.3327 " CO_2 and 0.1273 gm. H_2O.
0.300 " " " 0.0660 " AgI (glycerol determination).

	C	H	N	P	Glycerol.
Found	59.86	9.40	1.82	3.70	8.65

Commercial Lecithin.

The same cephalin has also been obtained from a sample of commercial lecithin. This was found to consist of considerable egg oil, together with sphingomyelin, cephalin, and lecithin. The cephalin fraction, insoluble in alcohol at 60°, had the same composition as the two samples analyzed above, and figures need not be given.

Lead Salt.

The lead salt was prepared by the method given in our second paper and had the following composition:

0.500 gm. substance neutralized 3.01 cc 0.1 N acid.
0.300 " " gave 0.0262 gm. $Mg_2P_2O_7$.

	N	P
Found	1.05	2.43

Kidney Cephalin.

A review of the work on the lipoids of the kidney has been given by Forbes and Keith.[7] The most important and complete investigation is that of MacLean.[8] In the present work cephalin

[7] Forbes, E. B., and Keith, M. H., Phosphorus Compounds in Animal Metabolism, Wooster, 1914, 141.

[8] MacLean, H., *Biochem. Jour.*, 1912, vi, 333.

was obtained from the kidney by the same method as that used in the work on brain cephalin. The product insoluble in alcohol at 60° had the following composition:

0.500 gm. substance neutralized 6.08 cc. 0.1 N acid.
0.300 " " gave 0.0394 gm. $Mg_2P_2O_7$.
0.1050 " " " 0.2316 " CO_2 and 0.0840 gm. H_2O.

	C	H	N	P
Found.....	60.17	8.95	1.70	3.65

The same results were obtained when the dried kidney material was extracted with ether. Because of the difficulty of purifying cephalin in general, no other details of the work will be given here.

Liver Cephalin.

The first alcohol-insoluble lipoid isolated from the liver was called jecorin.[9] The widely divergent results obtained by various observers indicated clearly that this substance was a mixture.[10] Baskoff[11] obtained a cuorin-like substance which he called *heparphosphatide;* this had a N : P ratio of about 1 : 1.5. Still later Frank[10] obtained a compound which resembled cephalin in its composition and properties.

The liver was worked up in the same general way as the other organs. The ether-soluble material of the alcoholic extract was precipitated repeatedly with alcohol and then with acetone. The cephalin was then emulsified with water, precipitated with 10 per cent hydrochloric acid, the precipitate taken up in ether and repeatedly precipitated with dry acetone. Solutions in amyl alcohol or ethyl acetate deposited the cephalin upon cooling, without

[9] Drechsel, E., *Jour. f. prakt. Chem.*, 1886, xxxiii, 425. Baldi, D., *Arch. Physiol.*, Suppl., 1887, 100. Jacobsen, A., *Skand. Arch. f. Physiol.*, 1895, vi, 262. Manasse, P., *Z. f. physiol. Chem.*, 1895, xx, 478. Drechsel, *Z. f. Biol.*, 1896, xxxiii, 86. Henriques, V., *Z. f. physiol. Chem.*, 1897, xxiii, 244. Meinertz, J., *Z. f. physiol. Chem.*, 1905, xliv, 371. Siegfried, M., and Mark, H., *Z. f. physiol. Chem.*, 1905, xlvi, 492. Waldvogel and Tintemann, *Z. f. physiol. Chem.*, 1906, xlvii, 129. Mayer, P., *Biochem. Z.*, 1906, i, 81. Baskoff, A., *Z. f. physiol. Chem.*, 1908, lvii, 395; 1909, lxi, 426; 1909, lxii, 162.

[10] For table of analyses and discussion, see Frank, A., *Biochem. Z.*, 1913, l, 277.

[11] Baskoff, *Z. f. physiol. Chem.*, 1908, lvii, 395.

any purification (see Baskoff). The material had the following composition:

0.500 gm. substance neutralized 6.8 cc. 0.1 N acid.
0.300 " " gave 0.0404 gm. $Mg_2P_2O_7$.
0.1622 " " " 0.3789 " CO_2 and 0.1306 gm. H_2O.

	C	H	N	P
Found	60.33	9.01	1.86	3.75

This material thus has the composition of cephalin. However, when fractionation out of alcohol at 60° was tried, it was found that this material could be separated into two rather constant fractions. The alcohol-soluble fraction had the composition of cephalin, while the alcohol-insoluble fraction contained a higher nitrogen content. In one case we were able to obtain a N : P ratio of nearly 2 : 1. We are not prepared, as yet, to discuss the significance of this fraction.

The so called cephalin, obtained above, was dissolved in ether and poured, with stirring, into alcohol heated to 60°. The filtrate was concentrated, and the material again precipitated in the same way. The third precipitation gives a body with the same N : P ratio, both in the precipitate and in the filtrate. This substance has all the properties of cephalin.

0.500 gm. substance neutralized 5.9 cc. 0.1 N acid.
0.300 " " gave 0.0394 gm. $Mg_2P_2O_7$.
0.1532 " " " 0.3404 " CO_2 and 0.1260 gm. H_2O.

	C	H	N	P
Found	60.6	9.20	1.65	3.66

Lead Salt.

The lead salt was prepared as usual. The first analysis is of a sample which had been washed with acetone; the second, of a sample which had been extracted with boiling methyl alcohol.

I. 0.500 gm. substance neutralized 3.96 cc. 0.1 N acid.
0.300 " " gave 0.0250 gm. $Mg_2P_2O_7$.
II. 0.500 " " neutralized 3.77 cc. 0.1 N acid.
0.300 " " gave 0.0282 gm. $Mg_2P_2O_7$.

	Found: N	P
I	1.10	2.32
II	1.06	2.61

THE DETERMINATION OF UREA BY THE UREASE METHOD.

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(Received for publication, January 4, 1916.)

In a recent number of this *Journal* Fiske[1] recommends as an improvement to the accuracy of our technique for urea determination[2] the use of a slower rate of aeration in driving the ammonia over into the standard acid, and the consequent prolongation of the time for aeration to an hour, in order to insure complete absorption of the ammonia by the acid. This modification and the consequent loss of time, however, are unnecessary if the directions given in our paper are followed; the accuracy attainable is limited only by that of the measurements and standard solutions. The conditions necessary for both complete and rapid absorption were studied rather exhaustively in experiments which it did not seem, at the time, essential to publish, but it now appears that certain of the principles on which our procedure was based must be stated more clearly.

1. One of the important factors in complete absorption is the height of the absorbing column of standard acid. We utilized as containers of the absorbing solution test-tubes of 100 cc. capacity and the usual shape (about 25 x 200 mm.). In such tubes 25 cc. of acid make a column 50 to 60 mm. high. Instead of these Fiske used "bottles," in which the column of acid is presumably much shorter. This change is the most obvious reason for the longer period of aeration required.

[1] Fiske, C. H., *Jour. Biol. Chem.*, 1915, xxiii, 455.

[2] Van Slyke, D. D., and Cullen, G. E., A Permanent Preparation of Urease, and Its Use in the Determination of Urea, *Jour. Biol. Chem.*, 1914, xix, 211.

2. Another point which may bear expansion is the reason for our statement (page 221): "If one uses *a moderate current of air for the first minute* every particle of ammonia is absorbed."

The necessity for caution at the start is that, with a given air current, the rate at which ammonia is carried over at the beginning of the aeration is enormously faster than afterwards. Consequently what caution is required should be concentrated on retarding the first burst of ammonia evolution. After that is over, it is almost impossible, with the apparatus and conditions described in our paper, to drive the ammonia over into the acid so fast that any of it escapes absorption. It does no harm, of course, to prolong the period of relatively slow aeration, but it is unnecessary. It is essential only that the fact be kept in mind that the first stage of aeration is the one that requires all the caution in regulating the air current. After this period is past, it is only a waste of time not to use as fast a current as is possible without loss of liquid from spattering. A much faster current can be used with a drop of caprylic alcohol to prevent foaming than with any other practicable agent, such as kerosene, for this purpose with which we are acquainted. We usually employ an air current of about 5 liters per minute, which insures complete aeration of the ammonia in 15 minutes.

Experiment 1.—The relatively high rate at which ammonia is evolved at the beginning of the aeration, and the constantly decreasing rate during the later stages, are indicated by the following experiment. 5 cc. portions of 0.0991 N ammonium sulfate (5 cc. = 24.78 cc. 0.02 N ammonia) were measured into each of a series of tubes arranged for aerating as illustrated on page 217 of our former paper, with the addition that a second absorption tube, containing 1 cc. of 0.01 N acid plus 10 cc. of water, was inserted after each pair of tubes. 5 cc. portions of 1:1 potassium carbonate solution were added, and the aeration was conducted with an air current of 5.3 liters per minute from the start. The ammonia was absorbed into 30 cc. portions of 0.02 N acid. At different intervals the successive sets of absorption tubes were disconnected and the ammonia which had been driven over was titrated. The results are given in Table I.

The air current was determined by Kober's[3] scheme of measuring the time required to draw 10 liters of water from one bottle into another. The water levels in both were so arranged that positive water pressure during

[3] Kober, P. A., and Graves, S. S., *Jour. Am. Chem. Soc.*, 1913, xxxv, 1594.

the first half of the transfer was balanced by negative during the second; and the connecting tube was so large (1.5 cm. inner diameter) that the flow of water was not appreciably retarded.

The analyst will find it worth while to standardize in this manner the air current and calculate the length of time required to drive 100 liters of air through the apparatus; as this amount with the apparatus described in our paper appears sufficient, with a good margin of safety, to drive over all the ammonia.

TABLE I.

Time of aeration.	Volume of air passed.	0.02 N HCl neutralized in first receiving tube.	0.02 N HCl neutralized in second receiving tube.	Total HCl neutralized.
min.	*liters*	*cc.*	*cc.*	*cc.*
1	5.3	13.5	0.08	13.58
2	10.6	20.3	0.08	20.38
3	15.9	22.8	0.08	22.88
5	26.5	24.1	0.08	24.18
10	53.0	24.6	0.09	24.69
15	79.5	24.7	0.09	24.79
25	132.5	24.7	0.08	24.78

At the beginning of the aeration the speed of evolution was so great that more than 55 per cent of the total ammonia was driven over in the first minute, 0.32 per cent being driven past the first receiving tube and caught in the second. During the second minute 27 per cent of the total ammonia was driven over. None passed the first tube during the second or following minutes. It is evident, therefore, that if the rate of evolution during the first 2 minutes had been reduced by cutting down the air current to one-half, no ammonia at all would have been lost. As it was, with a full air current from the start, the loss was only 0.3 per cent of the total, 99.7 per cent being retained in the first receiving tube. The amount of ammonia aerated was equal to the maximum ordinarily obtained in urine analyses.

It is a point of interest that, air current and other conditions being constant, the rate at which ammonia is driven over is exactly proportional to the amount present. The rate of evolution follows the same law $\frac{dx}{dt} = k\ (a - x)$ as the rate of a mono-

molecular reaction. Thus, taking a (total amount of ammonia present) equivalent to 24.78 cc. of 0.1 N acid, x being the amount driven over after t minutes, we have:

TABLE II.

t	x	$k = \frac{1}{t} \log_e \frac{a}{a-x}$	$k\ (a-x)$ Calculated rate of aeration after t minutes.
	Cc. 0.02 N NH_3		Cc. 0.02 N NH_3 per min.
min.			
0			20.2
1	13.58	0.794	9.0
2	20.38	0.867	3.6
3	22.88	0.857	1.5
5	24.18	0.744	0.5
Average		0.82	

The value, 0.82, of k indicates that at any given moment ammonia was being removed at the rate per minute of 82 per cent of the ammonia present.

The figures in the last column are calculated from the velocity equation $\frac{dx}{dt}$ = rate of aeration = $k\ (a - x) = 0.82\ (24.78 - x)$. They show that after 1 minute the rate of aeration had fallen parallel with the still remaining amount of ammonia, $a - x$, to less than half the initial rate, and that after 2 minutes the rate had fallen to one-sixth the initial velocity.

Experiment 2.—In order to ascertain the effect of using a half-speed air current during the first minute, four sets of tubes were aerated for 15 minutes in precisely the same manner as in the above experiment, except that during the first minute an air stream of 2.6 liters per minute was used, the 5.3 liter current being applied after 1 minute's aeration. The amounts of 0.01 N acid neutralized in the guard tubes were 0.04 to 0.05 cc., equivalent to 0.02 to 0.025 cc. of 0.02 N acid, or 0.08 to 0.10 per cent of the total ammonia, 99.90 to 99.92 per cent being absorbed by the 0.02 N acid in the first receiving tubes.

As an error not exceeding 1 part per 1,000 may be regarded as negligible for most purposes, we believe that no alteration need be made in the directions in our first paper for the technique of

aeration. If the analyst wishes, however, to make sure that even less than 1 part of ammonia per 1,000 escapes absorption, he may continue the half-current for 2 minutes instead of 1.

3. Since our former publication we have found, like Fiske, that addition of potassium carbonate in approximately saturated solutions, to set free ammonia for aeration, is preferable to its addition in solid form, and have privately recommended this modification. The difference is one of convenience rather than accuracy. Contrary to Fiske's assumption, the increase in volume of liquid from 5 cc. to 10 cc. does not significantly retard the evolution of ammonia during aeration. As we stated (page 221) after systematic tests, "The volume of the solution (in a 100 cc. test-tube) from which ammonia is being removed can be varied from 5 cc. to 25 cc. without appreciably altering the time required to drive off the ammonia," as long as the solution contains potassium carbonate in a concentration of at least 1 gm. to 2 cc. of solution.

4. Although, as we showed and Fiske repeats, urease in water extracts containing primary and secondary phosphate preserves most of its activity for several weeks when kept at 0°, ammonia is likely to form in such extracts, even at 0°, and in sufficient amounts to cause relatively large errors, particularly in blood analyses. The loss of activity also is rather insidious, for the reason that an extract which has remained for a long time almost without change loses activity at an unexplainably rapid rate after it has once begun to deteriorate. The safest way is to use a standardized preparation of dry urease, and prepare fresh solutions daily as required.

5. We have found it necessary, especially in blood analyses, to correct for ammonia in the reagents. The chief source of the correction is the potassium carbonate. Blank determinations are run on every lot of reagents, using 1 cc. of 10 per cent urease solution, 5 cc. of the 0.6 per cent phosphate solution, and 10 cc. of saturated potassium carbonate solution, and aerating into 0.01 N acid. The correction obtained has been as high as 0.12 cc. of 0.01 N acid, but with the carbonate now in use (Merck's U. S. P. viii) is 0.05 cc. of 0.01 N acid.

6. In closing we mention one essential precaution which we have always observed as a matter of course, but neglected to state in our original paper. As a result at least one colleague

has had much unnecessary trouble. If a test-tube and stopper are used as container, first for the strong carbonate solution, then, during a subsequent aeration, for the 0.02 N or 0.01 N acid solution, the tube or stopper, no matter how thoroughly washed with distilled water, is likely to carry a trace of alkali into the highly dilute acid and appreciably affect results. Therefore separate sets of tubes and stoppers must be employed as containers for the standard acid, and these must not be used for alkaline solutions. In case a tube that has been used for an alkaline solution is employed for standard acid, both tube and stopper are washed first with dilute acid, then with water.

It may be well also to call attention to the fact that the titration value of 0.01 N or 0.02 N alkali in ordinary glass vessels increases perceptibly from week to week, because the solution gradually dissolves more alkali from the glass. This action is particularly rapid in such a long narrow vessel as a burette. Alkali of 0.01 N concentration may appreciably increase its titration value as the result of standing for even a day in a burette.

STUDIES ON THE PHYSIOLOGY OF REPRODUCTION IN THE DOMESTIC FOWL.

XIV. THE EFFECT OF FEEDING PITUITARY SUBSTANCE AND CORPUS LUTEUM SUBSTANCE ON EGG PRODUCTION AND GROWTH.

By RAYMOND PEARL.

(From the Biological Laboratory of the Maine Agricultural Experiment Station, Orono (Paper No. 91), and the Organotherapeutic Laboratory of Armour and Company, Chicago.)

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I.

In the preceding paper in this series[1] it was shown that the injection of the substance or extract of the anterior lobe of the pituitary body into the peritoneal cavity of the domestic fowl failed entirely to activate the completely resting ovary. On general grounds, as set forth in the paper cited, there appeared to be many reasons for thinking it probable that pituitary substance might activate the ovary. On those grounds it was decided to extend the experiments with this substance somewhat farther, and along a different line. It is a well known fact, of course, that the specific physiological effects of certain endocrinal glands are produced when the gland substance is fed. Perhaps the most conspicuous example of this is found in the case of thyroid gland therapy. On the other hand, it is not to be forgotten that the products of endocrinal glands normally have a parenteral introduction into the body fluids. It is this consideration which has made intraperitoneal or intravenous injection the method of choice in the writer's experiments in the field hitherto.[2]

Because of the negative results with pituitary substance in the injection experiments it seemed worth while to try administration

[1] Pearl, R., and Surface, F. M., *Jour. Biol. Chem.*, 1915, xxi, 95.

[2] Pearl and Surface, *Jour. Biol. Chem.*, 1914, xix, 263; 1915, xxi, 95; *Maine Agricultural Experiment Station, Report for 1915*, 65.

per os. It is the purpose of this paper to record the results of certain experiments by this method.

The general plan of the first series of experiments to be tried was the following. With a group of hens sufficiently large to give reliable results, at a time in the laying year when under normal circumstances the rate of egg production is declining, will an administration of pituitary substance (anterior lobe) *per os* bring about an increase in the rate of fecundity? The experiment was carried out with three pens, each containing fifteen pure-bred barred Plymouth Rock hens, approximately 15 months of age at the beginning. In sorting the birds for the experiment into the three pens the attempt was made to get as even a distribution as possible, so that those in one pen should be substantially equal to those in the other pens in respect to innate laying ability, physiological condition at the time, body weight, breed, and general disposition.

To each bird in two of these pens (Pens A and B) was administered each day during the experiment (see below for dates) a No. 2 gelatin capsule filled with some mixture of pituitary body (anterior lobe) substance and milk sugar, or milk sugar alone. The data on the mixtures used and dates fed are as follows:

Pen A. Pituitary Pen.—July 10–24. Capsules filled with a mixture of three parts by weight of lactose and one part by weight of pituitary body (anterior lobe) substance. Average weight of filled capsule, 0.25 gm. This means, on the average, 0.0625 gm. desiccated anterior lobe substance per bird per day during this period.

July 25–Aug. 10. Capsules filled with a mixture of three parts lactose and two parts anterior lobe substance. This doubled the dose of the latter substance.

The pituitary substance used was the same as that used in the preceding experiments,[1] and prepared in the Organotherapeutic Laboratory of Armour and Company, especially for this work. Its preparation has been described in the paper cited and need not be repeated here.

Pen B. Lactose Control Pen.—July 10–Aug. 10. Capsules filled with milk sugar (lactose) only.

Pen C. Complete Control Pen.—July 10–Aug. 10. The birds in this pen received no capsules.

The results of the experiments are set forth in Table I and Fig. 1. All the birds in the experiment were trap-nested. These records are tabulated for (a) a preliminary period of 3 months and 9 days before the beginning of the pituitary feeding, (b) the experimental period, and (c) an after-period of 20 days. I have grouped the daily production into 5 day periods. The tabulated figures give the total number of eggs laid by each pen of birds in consecutive 5 day periods, the total production being supposed to be concentrated on the middle day of the period.

The data of Table I are shown graphically in Fig. 1.

TABLE I.

The Results of Feeding Pituitary Body (Anterior Lobe) Substance to Laying Hens.

Date.	Eggs laid in Pen A. (Pituitary.)	Eggs laid in Pen B. (Lactose control.)	Eggs laid in Pen C. (Complete control.)	Date.	Eggs laid in Pen A. (Pituitary.)	Eggs laid in Pen B. (Lactose control.)	Eggs laid in Pen C. (Complete control.)
Preliminary period.							
Apr. 3	49	44	52	July 2	49	43	39
" 8	47	45	55	" 7	54	40	37
" 13	45	46	46	Experimental feeding began July 10.			
" 18	46	48	43	July 12	43	33	35
" 23	49	40	37	" 17	51	35	40
" 28	45	33	32	" 22	45	35	40
May 3	56	41	34	" 27	43	40	35
" 8	56	42	31	Aug. 1	42	37	36
" 13	54	52	45	" 6	34	45	39
" 18	51	48	53	Experimental feeding ended Aug. 10.			
" 23	42	51	43	Aug. 11	40	36	34
" 28	55	54	42	" 16	39	38	46
June 2	49	48	43	" 21	43	28	39
" 7	51	42	44	" 26	36	32	40
" 12	42	37	40				
" 17	36	41	40				
" 22	39	42	41				
" 27	48	49	40				

Two birds in Pen A died during the experiment; one on Aug. 4, the other on Aug. 20.

From this table and diagram it is clear that:

1. The general trend of egg production in all the forty-five birds in the experiment in the period from Apr. 1 to Sept. 1 is downwards. This is the normal course of egg production at this season of the year.[3] That the general trend is downward in

[3] Compare Pearl and Surface, *U. S. Dept. of Agriculture, Bureau of Animal Industry, Bull. 110*, pt. ii, 1911, 81.

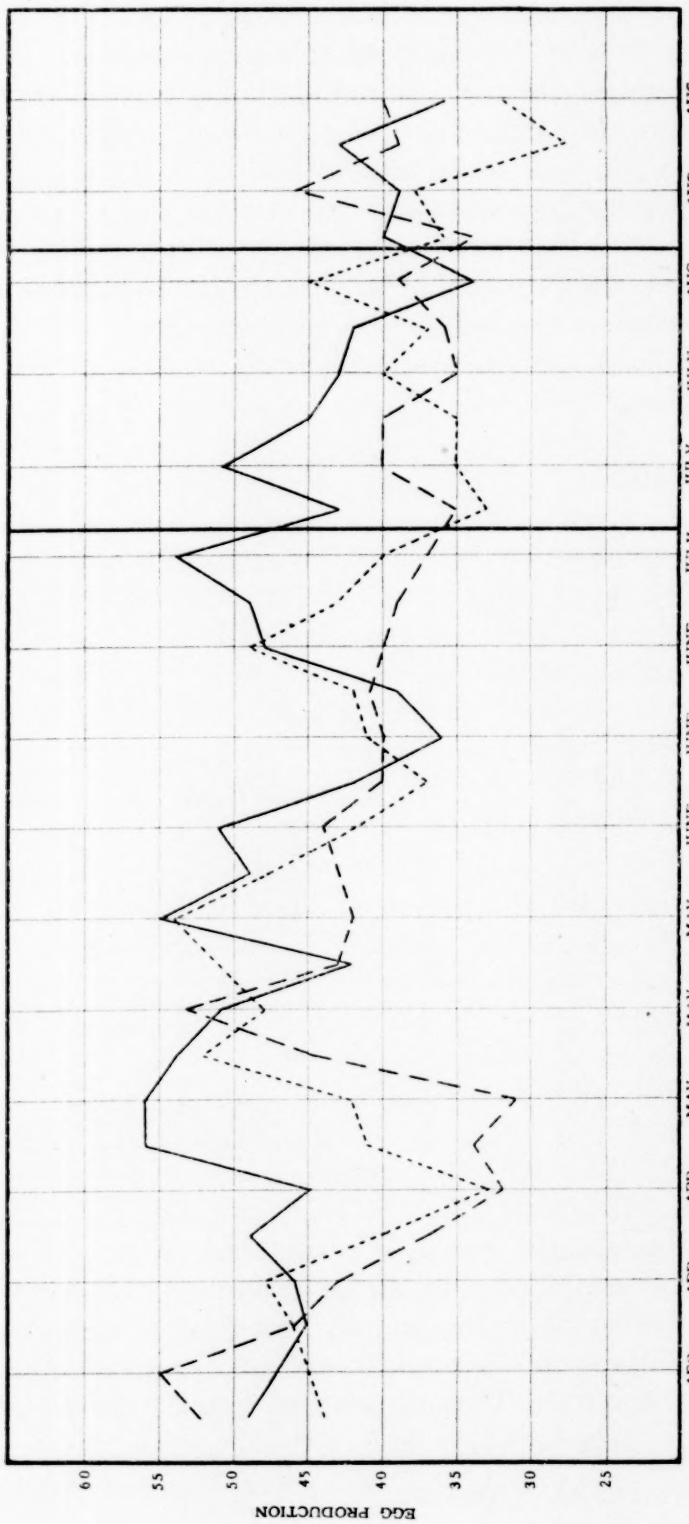

FIG. 1. Showing the egg production in experimental pens A, B, and C. Pen A, solid line; Pen B, dotted line; Pen C, broken line. The heavy vertical lines mark off the three periods of the experiment; *viz.*, the preliminary period, the feeding period, and the after-period.

these experimental flocks in the period covered by Table I is proved by the equations of the best fitting straight lines, fitted by the method of least squares, to these data. These equations are:

$$\text{Pen A} : y = 51.6184 - 0.3646\,x$$
$$\text{Pen B} : y = 47.6276 - 0.2400\,x$$
$$\text{Pen C} : y = 44.4207 - 0.2400\,x$$

where y denotes eggs produced, and x days after Apr. 1. The x term in all three equations is negative, demonstrating the general downward trend of the production, apart from the minor fluctuations.

2. The pituitary-fed birds fell off in production steadily during the period of experimental feeding (July 10–Aug. 10). For some weeks before, and at the beginning of the feeding period the Pen A birds had been laying at a somewhat higher rate than either Pen B or C. This difference was lost during the experimental feeding period, so that by Aug. 6 all the pens were laying at approximately the same rate.

3. There is not the slightest evidence, either from the figures presented above, or from the behavior or appearance of the birds during the experiment that the feeding of pituitary substance affected egg production in any way, either favorably or unfavorably. The results of this experiment thus confirm and extend the earlier work of this laboratory on pituitary substance.

4. Incidentally the figures and diagram show clearly the grave danger of error which inheres in drawing positive conclusions from minor fluctuations in an egg production curve, even in the presence of careful controls. Suppose, for example, that in the present instance the feeding of pituitary had begun about June 12, instead of on July 10 as was the fact. Immediately after June 12 the Pen A birds entered upon a period of heightened production, which was only to a small degree paralleled in the laying of the control pens B and C. One would have been very likely to conclude that this rise was due to the feeding of pituitary substance, had pituitary been fed at that time. Evidence of the favorable action of any agent on egg production needs careful scrutiny before it can be accepted. In particular the nature and amount of the change supposed to have been induced by the agent applied can be correctly and fairly interpreted only when account is

taken of the production curve of the experimental birds, and an equally large lot of control birds, over a preliminary period of at least 2 or 3 months. These precautions are often overlooked and quite erroneous conclusions reached by inexperienced students of egg production (see below).

II.

Another line of investigation which seemed worth undertaking was to determine whether the initial activation of the ovary could be accelerated by means of pituitary substance. A pullet of a good producing strain hatched at the proper time and well grown will ordinarily begin to lay when from 5 to 6 months of age. Some are more precocious and some less so, but, in general, the figures given represent a fair average. Can such pullets be brought to sexual maturity and laying any earlier if regularly fed pituitary substance?

To try this matter the following experiment was carried out. Forty-five pure-bred barred Plymouth Rock pullets, all hatched the same day (Apr. 29, 1915), were divided into three lots of fifteen each. They were so chosen that the total weight of the three lots, and thus the average weight per bird, were identical at the beginning of the experiment. Great pains were taken to get birds of the same stage of maturity and physiological development, as far as could be determined. Each bird in one lot (A) received *per os* 0.082 gm. per day of pituitary body (anterior lobe) substance. This was administered in No. 2 gelatin capsules with lactose as a diluent. Each bird in the second lot (B) received *per os* the same amount (0.082 gm.) per day of desiccated corpus luteum substance from pregnant cows, again administered in No. 2 gelatin capsules with lactose as a diluent. Finally Lot C was a control, the birds receiving no capsules. All the birds were housed in the same house, and given the same food and care except for the capsule feeding as above noted.

The experiment began Aug. 13, 1915. The growth of the birds is shown in Table II.

The essential facts brought out by this table are shown graphically in Fig. 2.

From the table and diagram it appears that:

1. Both pituitary body (anterior lobe) substance and corpus luteum substance retarded the growth of the birds in this experiment. Throughout the experiment all the birds were in a perfectly healthy and active condition. It was impossible to detect any difference in this respect among the three lots. There appears to be no means of accounting for the growth differences observed except as a result of the organ substances fed.

2. The retardation of growth in the case of the birds fed corpus luteum substance (Lot B) is greater than that in the lot fed pituitary substance (Lot A). The differences are very small and not separately significant until the weighing of Sept. 11 and later, from which time they are relatively large.

TABLE II.

Showing the Weights at Various Intervals of Birds Fed Pituitary Body (Anterior Lobe) Substance and of Those Fed Corpus Luteum Substance.

Date.	Lot A. (Pituitary.)		Lot B. (Corpus luteum.)		Lot C. (Control.)	
	Total weight.	Mean weight per bird.	Total weight.	Mean weight per bird.	Total weight.	Mean weight per bird.
	kg.	*gm.*	*kg.*	*gm.*	*kg.*	*gm.*
Aug. 13. Initial weight.	16.78	1,112.2	16.78	1,112.2	16.78	1,112.2
" 28	22.09	1,472.6	21.91	1,460.6	22.14	1,475.7
Sept. 4	23.81	1,578.6	23.59	1,571.1	24.18	1,611.8
" 11	24.68	1,645.0	24.22	1,614.8	25.40	1,693.4
" 18	25.72	1,714.6	25.45	1,696.4	26.81	1,787.2
" 25	27.12	1,808.3	25.63	1,708.5	28.26	1,883.9

3. After 43 days of organ substance feeding of birds starting at the same body weight, the birds fed pituitary substance average 4.01 per cent less in body weight, and those fed corpus luteum substance average 9.31 per cent less in body weight, than normal controls.

A parallel experiment to that reported above has been run with male birds and gave essentially the same results. Since the numbers used were too small to give reliable figures they are not published.

Leaving now the matter of growth in body weight we may turn to a consideration of the attainment of sexual maturity in these three lots of birds. In handling the birds for weighing

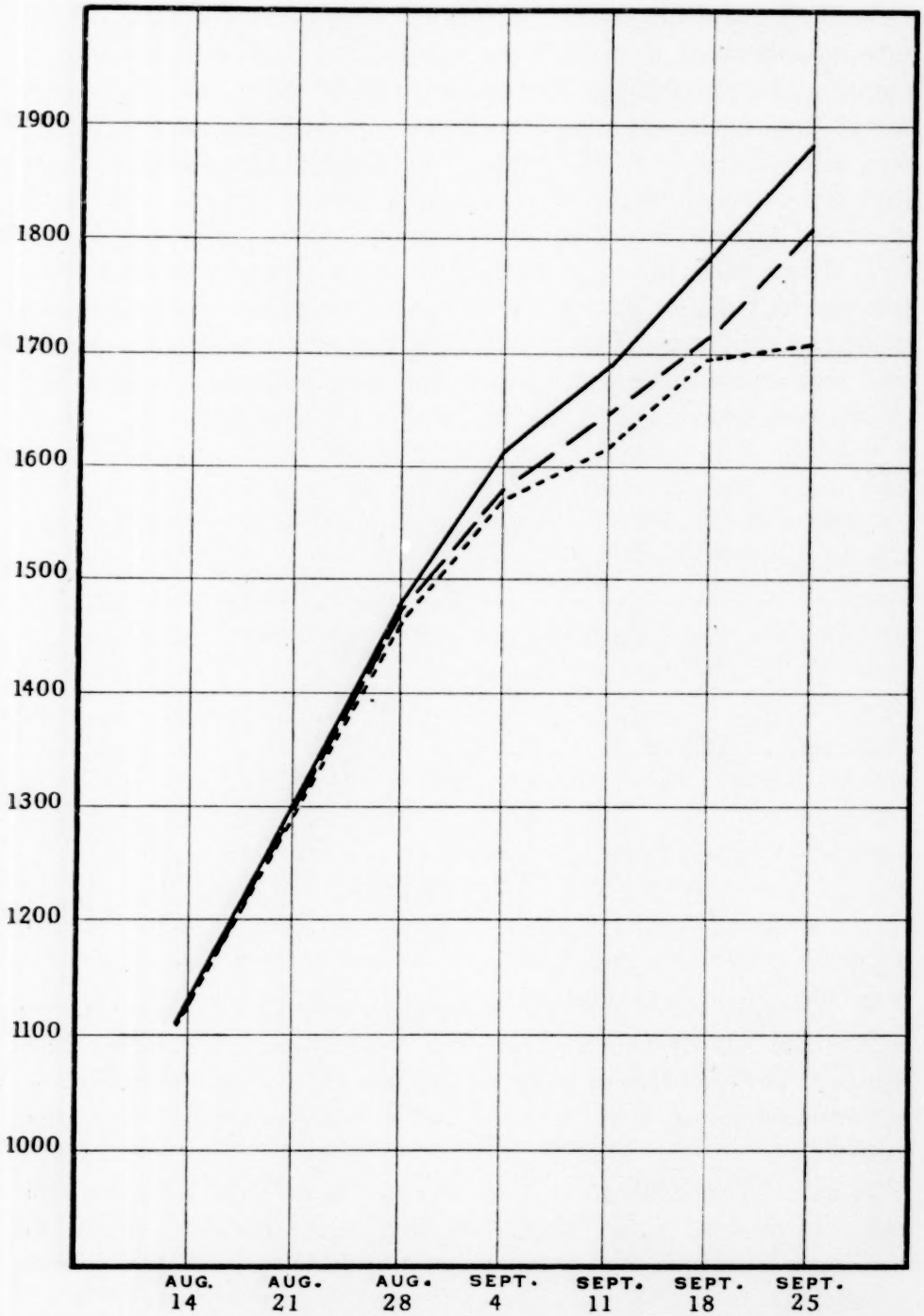

FIG. 2. Diagram showing mean weight per bird at successive dates following the feeding of pituitary substance and corpus luteum substance. Solid line, Lot C (control); broken line, Lot A (pituitary); dotted line, Lot B (corpus luteum).

and making general observations upon them no essential differences were detected.

The facts regarding onset of egg laying activities are shown in Table III.

From this table it is evident that there was practically no difference in the three lots of birds in respect to time of beginning laying. The small differences exhibited cannot be regarded as significant. Whether there would be a difference in average egg production in the several lots over a long period of time cannot be told from the present data, as the experiment had to be ended on Sept. 26.

TABLE III.

Showing Beginning Egg Production of Birds Fed Different Organ Substances as Indicated.

Date.	Eggs laid by		
	Lot A. (Pituitary.)	Lot B. (Corpus luteum.)	Lot C. (Control.)
Sept. 16	—	1	—
" 17	—	1	—
" 18	1	1	—
" 19	1	1	—
" 20	1	1	—
" 21	—	—	1
" 22	1	—	—
" 23	—	—	1
" 24	—	—	1
" 25	—	1	—
" 26	1	1	1

Several interesting points are brought out by this experiment. In the first place the results with pituitary body substance confirm all our earlier work with this substance as far as activation of the ovary is concerned. There is no evidence that the administration of pituitary substance hastened in any way the initial activation of the pullet ovary. The pituitary birds did not in fact begin to lay so soon as those receiving corpus luteum substance, though the 2 day difference between the lots cannot be regarded as significant. We have now tested the effect of pituitary (anterior lobe) substance on the ovary in three different physiological states; *viz.*, (a) completely resting adult ovary dur-

ing molt, (b) adult ovary in laying condition but with declining fecundity[4] rate, and (c) the inactive, immature ovary of the young pullet. In none of these physiological states has there been the slightest evidence that the pituitary has activated or accelerated the activity of the ovary in any manner or degree.

In the second place it appears that both pituitary (anterior lobe) substance and corpus luteum substance retard growth in the chick, but without affecting the attainment of sexual maturity (egg laying). The pullets in Lots A and B began to lay at the same time they would have had they not received organ substance, but their body weight at the onset of laying was from 4 to 9 per cent smaller than it would normally have been. These results are of interest in connection with the experiments of Gudernatsch[5] in feeding thyroid and thymus substance to growing tadpoles, though there is no evidence in the present experiments of differentiation being accelerated. It merely is not retarded, while body growth is retarded by pituitary and corpus luteum substance. It will be a matter of much interest to extend the period of feeding these substances, particularly corpus luteum, into earlier life. The writer proposes to do this next year. For students of growth a means is afforded in corpus luteum feeding of notably retarding body growth without disturbing the normal physiology or physiological development.

The results on growth in Lot A confirm the findings of various other workers who have studied the effect of pituitary substance on growth in other animals than the chick. Cushing[6] found that repeated injections of anterior lobe material caused a definite loss of weight. Sandri[7] found in mice and guinea pigs that pituitary feeding, or administration of an emulsion of the gland by injection, resulted in diminished growth. Aldrich,[8] apparently contrary to his expectations, found that when anterior lobe substance was fed to white rats it caused a definite and distinct re-

[4] Compare Pearl, *Jour. Exper. Zool.*, 1912, xiii, 155, for definition of "fecundity" as used in papers from this laboratory.

[5] Gudernatsch, J. F., *Arch. f. Entwcklngsmechn. d. Organ.*, 1912–13, xxxv, 457; *Am. Jour. Anat.*, 1913–14, xv, 431.

[6] Cushing, H., *Jour. Am. Med. Assn.*, 1909, liii, 249.

[7] Sandri, O., *Arch. ital. di biol.*, 1909, ii, 337.

[8] Aldrich, T. B., *Am. Jour. Physiol.*, 1912–13, xxxi, 94.

tardation of growth. He fed 30 mg. per day for a period of 100 days and at the end of the period the body weight of the experimental rats was, on the average, 16.45 per cent in defect of that of the controls. The posterior lobe of the gland produced no effect on growth. Schäfer[9] appears to have been the only worker who has failed to find the retarding effect of anterior lobe substance upon growth, and his results have not been confirmed by subsequent workers.

I am not able to find that the similar but much more marked retarding effect of corpus luteum substance upon growth has hitherto been noted in the literature. Sack[10] has studied in rats the effect on general metabolism of feeding corpus luteum substance, and reports the finding of specific effects in the female. He has no data upon growth, however, and the period of feeding was so short and the number of animals used so small as to make his conclusions doubtful.

III.

While the investigations reported in this paper were in progress a paper by Mr. L. N. Clark[11] appeared dealing with the effect of pituitary body substance upon egg production in the domestic fowl. This author reports striking positive results in the direction of increasing egg production, following the feeding of anterior lobe. He attributes the difference between his results and those of Pearl and Surface to the fact that he used the gland substance of young animals while we used that from adults.[12] Whether or not the age of the animal furnishing the gland accounts for the difference in results between Mr. Clark and this laboratory remains to be shown. Pending the results of investigation directed towards elucidating that point, however, the present writer desires to express the opinion that Mr. Clark's figures do not appear to demonstrate completely his conclusion that the pituitary substance stimulated the ovary. Before this conclusion

[9] Schäfer, E. A., *Proc. Roy. Soc., Series B*, 1909, lxxxi, 442.

[10] Sack, W. T., *Arch. f. exper. Path. u. Pharm.*, 1912, lxx, 293.

[11] Clark, L. N., *Jour. Biol. Chem.*, 1915, xxii, 485.

[12] Mr. Clark's assumption that we used a commercial preparation is gratuitous. The material was especially prepared for us by Dr. Frederic Fenger.

could be accepted by anyone inclined to be critical it would be necessary to show by careful analysis of the egg production curves of the flocks used, extending over several months at least, that the fluctuations observed are not such as would have been as likely as not to occur had no experimental substance been fed. Mr. Clark's figures for his experimental pens contain no fluctuations greater, more sudden in onset, or more prolonged in duration, than the writer has repeatedly observed in the Maine Station flocks, *not subjected to any experimental treatment whatever at the time*. If the critical reader will study Fig. 1 of this paper, and then go over Mr. Clark's figures, plotting them as percentage productions so as to get experimental and control pens to the same base, he will get some idea of the extreme difficulty of adducing proof, or anything like proof, that a change upward in an egg production curve following the administration of an experimental agent is caused by that agent. Mr. Clark from his experience as a poultryman recognizes some of these difficulties, and properly points out that such experiments can only be considered critical when the general trend of the production curve is declining (p. 486).

One further point in which Mr. Clark's experiments appear to me to be open to serious criticism is in the fact that nowhere does he have any proper controls. In the first experiment where the thirty-five birds were fed individually flocks of 657 and 431 are cited as controls. But anyone who has had experience in experimental work with poultry knows that a reliable control can only be had by using a flock of the same size as the experimental flock, in which the individual birds are carefully selected so that they shall be as nearly as possible like the experimental birds in respect to all their characteristics including breed, age, body weight, stage of maturity, physiological condition, health, disposition, etc. Mr. Clark, as a practical poultryman, must surely appreciate these points, and as this first paper of his is said (p. 491) to be only preliminary, we may in his future experiments confidently look for more careful and critical attention to these safeguards which are essential to make a poultry experiment trustworthy. We shall look forward with great interest to the results of Mr. Clark's further and more critical experiments. Certainly the conclusions which he reaches are of great theoretical and practical interest, especially if confirmed by further critical work.

SUMMARY.

1. Feeding the desiccated substance of the anterior lobe of the pituitary body of cattle to hens in laying condition but at a time of year when the rate of fecundity is declining, does not stimulate the ovary to an increased rate of production.

2. Feeding the same substance (pituitary body, anterior lobe) to growing pullets does not bring about any earlier activation of the ovary than occurs in normal control pullets not fed this substance.

3. The anterior lobe of the pituitary body from cattle when fed to growing chicks is accompanied by a distinct retardation in growth in body weight. This confirms for the chick the results which have been obtained with this substance by other investigators in mammals.

4. The feeding of the desiccated substance of corpus luteum brings about a retardation of growth about twice as great in amount as that following pituitary feeding, as above noted.

5. Neither pituitary substance (anterior lobe) nor corpus luteum substance when fed to laying pullets causes any retardation in the attainment of sexual maturity as indicated by the laying of eggs. The birds so fed begin to lay eggs at the same age, but at a smaller body weight than the normal controls.

6. Some of the essential safeguards for the critical conduct of organ substance experiments with poultry are discussed.

CHANGES IN THE WEIGHT AND COMPOSITION OF FASTING LOBSTERS.[1]

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A statement in the literature[2] that lobsters retain their original weight even after several months of fasting led to the present investigation. The constancy of body weight may be regarded as an index of physiological equilibrium, and its fluctuations indicate the internal condition of the organism. By the fall in body weight the normal course of a fast may be followed from day to day, just as normal growth may be studied by the periodic gains of the organism.

As far as we know, all organisms, temporarily deprived of nourishment, diminish progressively in mass, and this is registered by changes in weight. The observation of the constancy of the weight of fasting lobsters seemed, therefore, worthy of investigation. The two sides of the problem, *i.e.*, the changes in weight and composition of the fasting lobsters, have been studied simultaneously.

Preparatory to weighing, the lobsters were wrapped in towels and the remaining traces of moisture were wiped off thoroughly from every portion of the body. Each animal was then placed in an aluminum can in which it was carefully weighed to 0.01 gm. The can was then weighed without the lobster, as the animals frequently excreted a small quantity of fluid during the weighing, and the weight of the lobster was calculated. The lobsters were kept in flat glass dishes partially filled with filtered

[1] Published by permission of the Commissioner of Fisheries.

[2] Moore, B., Edie, E. S., and Whitley, E., *Report on the Lancashire Sea-Fisheries*, 1913, xxii, 297.

sea water, which was continuously aerated. The water was changed every second day, and the weighing was made every 2 weeks. The experiment lasted for nearly 2 months.

The data relating to the changes in total body weight are recorded in Table I, in which the percentage of the biweekly change and the per cent of the loss suffered in the 56 days of inanition are given. It will be observed that the losses ranged from 0.10 to 1.89 per cent during the 2 week periods. On two occasions a slight increase of 0.3 per cent was observed. It will be seen from the table that all the lobsters lost weight, though this loss was extremely small. Thus in the 56 days of fasting they lost, on an average, 4.83 gm. or 2.89 per cent of their initial weight. In view of the facts recorded here it is hardly justifiable to speak of a constancy of weight of lobsters subjected to inanition. The question naturally arises as to whether the singularly low rate of loss of body weight indicates that the metabolic activity of these animals is very low, or that the actual loss sustained by the lobster is masked by secondary occurrences. The study of the composition of normal and starved lobsters gives a definite answer to this question.

The following methods have been employed in the investigation of the chemical composition. Both the control and experimental animals were rinsed with clean water, thoroughly dried, weighed, and after being cut in small pieces they were desiccated at about 100°C. When no further diminution in weight was found, the dry material, shell and all, was ground to a fine powder which was again dried in the oven until the weight remained constant. The dry residues from the different lobsters were combined, thoroughly mixed, and used for the various analyses. The determinations made on these composite samples were as follows: total ash, non-volatile inorganic portion, total nitrogen, glycogen, and the ether-, alcohol-, and water-soluble matter. In the latter two portions the nitrogen was likewise determined.

In determining the total amount of ash a weighed portion of the dry material was at first charred over a low flame. This preliminary heating was never carried further than the incipient dull redness. The charred material was repeatedly extracted with boiling water until the test for chlorides was negative. The extracted material was then completely incinerated while the extract was evaporated. The combined weight of the two is the total ash content. The non-volatile inorganic constituents were determined separately by incinerating the dry matter directly in a muffle.

The glycogen was isolated according to Pflüger, and after hydrolysis it was measured by Allihn's gravimetric method.

TABLE I.

Changes in Weight of Lobsters Fasting from July 12 until September 6.

No.	1		2		3		4		5		6		Average.	
Date.	Weight.	Bi-weekly loss.	Weight.	Bi-weekly loss.	Weight.	Bi-weekly loss.	Weight.	Bi-weekly loss.	Weight.	Bi-weekly loss.	Weight.	Bi-weekly loss.	Weight.	Bi-weekly loss.
	gm.	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*
July 12	175.50		168.33		143.87		189.85		165.15		162.35		167.51	
" 26	176.11	+0.35	167.29	−0.62	143.03	−0.60	189.20	−0.40	163.56	−0.97	159.69	−1.70	166.48	−0.62
Aug. 9	173.62	−1.42	165.67	−0.97	142.89	−0.10	187.85	−0.71	161.18	−1.46	159.31	−0.24	165.09	−0.84
" 23	173.19	−0.25	165.35	−0.20	141.35	−1.08	187.55	−0.16	161.72	+0.34	158.18	−0.80	164.56	−0.32
Sept. 6	171.31	−1.08	163.17	−1.32	138.68	−1.89	186.18	−0.73	160.77	−0.59	155.97	−1.39	162.68	−1.14
Total loss	4.19	−2.39	5.16	−3.07	5.19	−3.61	3.67	−1.94	4.38	−2.65	6.38	−3.93	4.83	−2.89

The extraction with ether, then with alcohol and water, was made on the same sample of dry material, of which about 20 gm. were used for each determination. We used ether redistilled over sodium, and the alcohol was prepared from absolute alcohol by boiling it for 2 days with lime and distilling it over lime. This alcohol was kept over anhydrous copper sulphate.

The extraction with alcohol, which followed the ether extraction, was made in the same Soxhlet apparatus. By wrapping the apparatus with a layer of cotton it was possible to raise the temperature of the extraction cup so that a continuous flow of hot alcohol was maintained for 48 hours.

The material thus treated with ether and alcohol was boiled several times with distilled water until the filtered extract was perfectly colorless. The combined watery extracts were condensed and made up to 200 cc. Aliquot portions of this served for the nitrogen determination, as well as for the organic residue. This was determined by evaporation to dryness and analyzing the residue for its total yield of ash by the above method. The amount of organic extractives was obtained by subtracting the weight of the ash from the total dry weight.

Table II consists of two parts: in one the percentage composition of the dry matter of both normal and fasting lobsters is given; in the other these data are recomputed for the fresh material. Normal lobsters have on an average 67.33 per cent of water, the individual variations ranging from 65.9 to 69.5 per cent. About two-thirds (64.3 per cent) of the dry matter is organic. Of the inorganic matter about three-fourths is non-volatile constituents, while the volatile material consists chiefly of chlorides. The ether, alcohol, and water extractives represent 2.92, 5.61, and 10.53 per cent of the dry matter, respectively. The alcohol-soluble moiety contains 7.9 per cent of nitrogen, while in the water extractives there is 10.89 per cent of nitrogen. In determining the protein content I have departed from the customary way of giving as protein the product of the total nitrogen by the factor 6.25, but the nitrogen extracted by the various solvents which is not of protein origin has been deduced from the total amount of nitrogen found in the dry material. From the results of our experiments we may safely say that Moore is probably wrong in stating that "there is very little nitrogen other than protein nitrogen."[1] In my experiments the alcohol- and water-extracted nitrogen forms 22 and 24 per cent, respectively, of the total nitrogen of fasting and normal lobsters.

With the correction as stated above, the protein content of normal lobsters is computed as 31.54 per cent. But even this figure

TABLE II.

Percentage Composition of Normal and Fasting Lobsters (Fresh).

	Dry matter.	Water.	Organic matter.	Total inorganic matter.	Non-volatile inorganic matter.	Total glycogen.	Ether extract.	Alcoholextractives.	Alcohol-extracted nitrogen.	Per cent of nitrogen in alcohol extract.	Water extractives.	Water-extracted nitrogen.	Per cent of nitrogen in water extract.	Total nitrogen.	Protein (non-extracted nitrogen × 6.25).	Undetermined.
Control (average of five).....	32.672	67.328	21.010	11.662	8.955	0.161	0.954	1.831	0.145	2.582	3.285	0.374	3.558	2.168	10.315	4.464
Fasting 56 days (average of five).....	21.374	78.626	10.766	10.608	8.938	0	0.051	0.361	0.019	1.100	1.248	0.171	2.924	0.957	4.793	4.313

Percentage Composition of the Dry Matter of Normal and Fasting Lobsters.

Control (average of five).....	64.305	35.695	27.409	0.494	2.919	5.605	0.443	7.904	10.528	1.146	10.891	6.636	31.544	13.215
Fasting (average of five).....	50.370	49.630	41.817	0	0.238	1.691	0.087	5.145	5.767	0.876	15.190	4.475	22.425	20.249

is probably too high, since some of the nitrogen considered as belonging to the protein is really a component part of the chitin of which the shell is largely made up. Chitin contains about 6 per cent of nitrogen (protein contains 16 per cent). Unfortunately the weight of the shells was not determined, and it is therefore impossible to compute even approximately the chitin nitrogen fraction. But it is clear that the amount of protein is less than that given in the table and that the undetermined portion, recorded in the last column, should be correspondingly higher than 13.2 per cent.

The composition of fasting lobsters, as compared with that of the normal lobsters, shows an unusually large increase in the proportion of water, which was increased to 78.63 per cent, with individual variations ranging from 77.6 to 79.7 per cent. An increased content of water has been generally observed in fasting organisms, but the change from 67.3 to 78.6 per cent, *i.e.*, by more than 11 per cent, must be regarded as entirely unparalleled in any other animal. The dry matter is made up of practically equal parts of organic and inorganic substance. About four-fifths of the latter is non-volatile, a proportion greater than that found in normal lobsters, which is due to the predominant loss of chlorides and possibly also carbonates. The ether-, alcohol-, and water-soluble materials show likewise a marked reduction to 0.24, 1.69, and 5.77 per cent, respectively. The diminution is particularly noticeable in the case of the ether-soluble substances, the quantity of which decreased to less than one-twelfth the normal content. In comparison with that, the alcohol and water extractives have diminished much less, the former from 5.6 to 1.7 per cent, and the latter from 10.5 to 5.8 per cent. The nitrogen content of these extractives has not diminished in the same ratio. In the alcohol extractives it has changed from 7.9 to 5.1 per cent, while in the water extractives it increased from 10.9 to 15.2 per cent, or nearly one and a half times. It follows, therefore, that in the fasting lobsters the non-nitrogenous fraction of the extracted material decreased more rapidly than the nitrogenous.

It will be observed, furthermore, that the glycogen has been exhausted so that in the samples analyzed, at any rate, nothing could be recovered. It is possible, of course, that a slight trace may still have been present in the entire lobster.

The undetermined moiety of the dry matter of fasting lobsters is 20.25 per cent, and this is considerably higher than in the normal animal. It has already been pointed out that the undetermined fraction is doubtless chitin, and this would suggest further that the percentage of chitin is greater in fasting lobsters, which may be due either to the fact that the amount does not diminish as rapidly as that of the proteins, or that it does not diminish at all. In this connection the per cent of undetermined material found in the fresh substance gains particular significance. It will be seen that this was 4.46 per cent in the normal and 4.31 per cent in the fasting animals. We shall return to this subject later on.

The analyses here recorded differ considerably in their results from those given by Moore and Herdman.[3] This may be partly accounted for by the consideration that these authors determined only the total nitrogen and, multiplying by the usual factor 6.25, expressed it in terms of protein. But apart from this they found in their lobsters a much higher proportion of fat, glycogen, and ash. Even in the series which fasted 235 days they still found 2.14 per cent of fat, whereas my lobsters contained barely over 0.2 per cent at the end of 56 days. The per cent of undetermined material is, therefore, much lower than in my experiments. But here again, since Moore and Herdman consider all the nitrogen of the dry matter of lobsters to be protein nitrogen and do not make any allowance for the chitin, it is obvious that the actual amount of undetermined matter must be greater than is shown in their tables.

With the data given in Table II we may now compute the average composition of the lobsters before and after the fast, in terms of the absolute quantities of the various components. This has been done in Table III. The interesting fact brought out by this comparison of the initial and final composition is that the water in the organism is not merely increased relatively, as has been ordinarily found in the case of starving animals, but that it increased *absolutely*. Thus it is computed that at the start there were 112.67 gm. of water per animal. The amount actually

[3] Moore, B., and Herdman, W. A., *Report on the Lancashire Sea-Fisheries*, 1913, xxii, 321.

TABLE III.

Average Composition, in Grams, of Lobsters before and after Fasting.

	Live weight.	Dry matter.	Water.	Organic matter.	Total inorganic matter.	Non-volatile inorganic matter.	Total glycogen.	Ether extract.	Alcohol extractives.	Alcohol-extracted nitrogen.	Water extractives.	Water-extracted nitrogen.	Total nitrogen.	Protein (non-extracted nitrogen × 6.25).	Undetermined.	Per cent of organic matter determined.
Initial (computed)..	167.34	54.67	112.67	35.16	19.51	14.99	0.27	1.60	3.06	0.24	5.51	0.63	3.63	17.26	7.46	78.78
Final (determined)..	162.58	34.75	127.83	17.50	17.25	14.53	0	0.08	0.59	0.03	2.03	0.28	1.56	7.79	7.11	59.95
Absolute loss in gm..	4.76	19.92	+15.16	17.66	2.27	0.45	0.27	1.51	2.48	0.21	3.48	0.35	2.07	9.47	0.35	
Loss in per cent.....	2.73	57.3	+13.46	50.22	11.62	3.03	100.0	94.80	80.84	87.20	63.17	55.59	57.11	54.75	4.69	

found at the end of 56 days of starvation was 127.83 gm., or 15.16 gm. more.

We may also point out the comparative constancy of the non-volatile portion of the ash. It was diminished by 0.45 gm., or 3.03 per cent, and it is doubtful if such a small diminution has any significance in view of the fact that the initial quantity is computed and not directly determined. It is probably more nearly true to assume that the non-volatile inorganic constituents remain practically unchanged through fasting.

It should further be observed that the various extractives suffer most heavily. Thus 94.8 per cent of the ether-soluble material is used up, while of the alcohol and water extractives 80.8 and 63.2 per cent, respectively, disappear. It is significant that the amount of material which remained undetermined in our analyses is nearly the same in the lobsters before and after the fast. The loss of 0.35 gm., or 4.69 per cent, does not seem large enough to merit much consideration, and, though it would not justify the assumption that this particular component does not undergo any change, it is clear that the change must be of slight importance.

If the different organic constituents are added together it is found that their weight makes up 78.78 per cent of the total dry matter in the normal condition, and only 59.95 per cent in the starved. At first thought this failure to recover more of the organic substance in spite of the more or less extensive analytical treatment would suggest that the analyses were faulty. I have assumed this attitude towards the results of the analyses, but the same data were obtained by repeating practically the whole work. The question has been cleared up, however, by the fact that chitin, which is apparently unaffected by inanition, forms a larger proportion of the fasting than of the normal lobster. It is of interest to observe that a substance comparatively rich in nitrogen and calorific value should remain untouched, while under the great stress produced by the inanition all other tissues of the organism contribute heavily to its maintenance. It raises the problem of the availability of glucosamines as a source of energy. This point will be studied in the future.

We shall now attempt to answer the question we have undertaken to solve at the beginning of this paper. A review of the

data in the last line of Table III shows that as far as the different organic substances of the lobsters are concerned the rate of their loss of weight was as great as in any other known instance on record. The changes we find here are in close agreement, for instance, with those which I observed several years ago in starving salamanders.[4] The actual loss in body weight suffered by the lobsters is masked by the imbibition of water. We can easily compute what the probable loss was at the end of 56 days of fasting. The loss found from the change of the total body weight was only 2.73 per cent. But let us suppose that no imbibition of water had taken place. Furthermore, with the knowledge gained from other studies of fasting organisms we may assume that at this particular phase of starvation the quantity of water in the body would have diminished about 33.3 per cent. Starting with a quantity of 112.7 gm. of water, this would have decreased to 75.1 gm. at the end of 56 days of fasting. The weight of the lobsters would therefore have been 109.8 gm., and the loss 34.4 instead of 2.73 per cent. The hard shell protecting the entire body of the lobster and forming a solid supporting structure prevents the cells of the soft tissues from shrinking as they ordinarily do under the influence of inanition when the metaplasmic inclusions are being gradually used up. This may explain the extraordinary extent of the imbibition of water by the tissues as their reserves are being exhausted. The relative increase in the water content of the body which invariably occurs in inanition is unquestionably to reduce the concentration of the body juices. But the great absorption of water by the tissues of starving lobsters is the result of primarily mechanical factors, the tissues imbibing an excess of water in the manner of a sponge.

My thanks are due to Mr. E. W. Fuller for the assistance which he rendered in the course of these experiments.

[4] Morgulis, S., *Arch. f. Entwcklngsmechn. d. Organ.*, 1911, xxxii, 169.

THE LEWIS AND BENEDICT METHOD FOR THE ESTIMATION OF BLOOD SUGAR, WITH SOME OBSERVATIONS OBTAINED IN DISEASE.

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A large number of methods have been described for the estimation of blood sugar. Although many of these determine the reducing power of the blood with a high degree of accuracy, they are almost all so technical as to require special equipment and training. S. R. Benedict, who introduced the most satisfactory qualitative and quantitative tests we possess for sugar in urine,[1] has performed, in collaboration with Lewis,[2] a similar service for the sugar of the blood. The general principles and technique of this method are far simpler than those of any method previously described, and it seems probable that it will completely replace other blood sugar methods for both scientific and clinical purposes. The method is dependent upon the fact that very small amounts of glucose react with picric acid and sodium carbonate to produce a red color (picramic acid). In the actual test the picric acid serves the additional purpose of precipitating the proteins of the blood.

R. G. Pearce[3] has recently suggested a modification of the method to overcome the necessity for evaporation required with the original method. For more than a year and half we have utilized a procedure which accomplishes this purpose more simply, we believe, than that described by Pearce. It is the purpose of the present paper to present briefly the technique which we have

[1] Benedict, S. R., *Jour. Biol. Chem.*, 1909, v, 485; 1911, ix, 57.

[2] Lewis, R. C., and Benedict, S. R., *Jour. Biol. Chem.*, 1915, xx, 61. The paper contains an excellent review of the older methods.

[3] Pearce, R. G., *Jour. Biol. Chem.*, 1915, xxii, 525.

employed, together with some of our observations on hyperglucemic conditions obtained with the method.

The Lewis-Benedict procedure calls for a blood dilution of 1:12.5. 2 cc. of blood are treated with 8 cc. of water to lake the corpuscles and then 15 cc. of saturated picric acid are added to precipitate the proteins. Pearce utilizes the same dilution, but to avoid evaporation, develops the color in the diluted solution with the aid of the higher temperature of the autoclave. Comparison is made with a picramic acid solution one-fourth as strong as that utilized by Lewis and Benedict.

In connection with a rather large number of estimations of creatinine and creatine in the blood,[4] it was found advantageous to utilize the same preliminary step for the determination of blood sugar. 5 or 6 cc. of blood are treated in a 50 cc. centrifuge tube with four volumes of water to lake the corpuscles and dilute the blood. To the mixture is then added sufficient dry picric acid (0.5 to 1.0 gm.) to precipitate the proteins and render the solution saturated.[5] The mixture is stirred at intervals until it is a light yellow, then centrifuged, and the supernatant fluid filtered through a small filter paper. 10 cc. of the filtrate are employed for the estimation of creatinine, 5 cc. for creatine, and 3 cc. for sugar. By employing 3 cc. of this filtrate from the blood diluted 1:5, the volume is sufficiently small to require no evaporation in the development of the color with the sodium carbonate. 3 cc. of this filtrate, the equivalent of 0.60 of 1 cc. of blood, are very close to the quantity employed by Lewis and Benedict—their 8 cc. are the equivalent of 0.64 cc. of blood. With normal blood this contains a quantity of sugar sufficient to give a depth of color at a 10 cc. dilution most suitable for comparison in the colorimeter. Heating a test-tube containing 3 cc. of the picric acid filtrate and 1 cc. of 20 per cent sodium carbonate in a beaker of boiling water for 15 minutes suffices for the development of the color, after which the tube is cooled and diluted to a volume suitable for colorimetric comparison.

[4] Myers, V. C., and Fine, M. S., *Jour. Biol. Chem.*, 1915, xx, 391. Myers, V. C., and Lough, W. G., *Arch. Int. Med.*, 1915, xvi, 536. A description of these methods has been given in Myers, V. C., and Fine, M. S., with the collaboration of Bailey, C. V., and Gorham, F. D., Chemical Composition of the Blood in Health and Disease, New York, 1915.

[5] Compare Folin, O., *Jour. Biol. Chem.*, 1914, xvii, 475.

In our earlier determinations the Duboscq colorimeter was employed, but more recently we have used the Hellige (Autenrieth and Koenigsberger) instrument, which is much more rapid and quite as satisfactory. As a standard color, picramic acid has been employed since Dr. Benedict suggested its use to us some months prior to the publication of his paper. We are further indebted to him for placing a supply of this chemical at our disposal.

Epstein[6] has suggested a modification of the Lewis-Benedict method whereby very small amounts of blood (0.2 cc.) are employed, this being obtained by puncture of the finger tip or lobe of the ear. Our experience has been that taking blood from a vein and thus securing a larger amount is more satisfactory and causes the patient less discomfort. Where the estimation of the blood sugar is not a part of a general blood examination in which 25 to 30 cc. are withdrawn, we have found the use of a 2 cc. glass barrel syringe fitted with a fine irido-platinum or steel needle to be very satisfactory. In this way blood has been taken from patients every 15 minutes over the greater part of a day.

Method.

About 2.5 cc. of blood are drawn into a glass syringe, which has been washed out with a drop or two of 20 per cent potassium oxalate to prevent clotting, and then delivered into a test-tube containing a very little powdered potassium oxalate. With an Ostwald-Folin pipette 2 cc. of the blood are pipetted into a cylindrical centrifuge tube of 15 to 20 cc. capacity graduated to 10 cc. The pipette is thoroughly washed out by pipetting 8 cc. of water into the tube. The blood is thus diluted 1:5 and the corpuscles are thoroughly laked. About 0.2 gm. of dry picric acid is now added and the material thoroughly mixed with a glass stirring rod. After the protein is completely precipitated and the solution has become saturated with picric acid, it is allowed to stand several minutes with occasional stirring. The tube is now centrifuged and the supernatant liquid filtered into a dry test-tube through a small 4 cm. filter paper. 3 cc. (sufficient material is generally available for duplicate determinations, if de-

[6] Epstein, A. A., *Jour. Am. Med. Assn.*, 1914, lxiii, 1667.

sired) of the filtrate are pipetted into a tall, narrow test-tube, graduated to 3, 4, 10, 15, and 20 cc., 1 cc. of 20 per cent sodium carbonate is added, and the tube heated in a beaker of boiling water for 15 minutes. At this stage the color is completely developed though uninfluenced by prolonged heating. The solution is now cooled to room temperature and water added to bring the volume up to 10, 15, or 20 cc., etc., according to the depth of color. The solution is thoroughly mixed and compared in the Hellige colorimeter with the wedge of standard picramic acid.[7] The scale is most accurately read between 40 and 65. This would require a dilution to 10 cc. for normal blood. If the standard Hellige instrument is used with 0 at the top of the scale,[8] the value of the colorimetric reading in percentage of blood sugar may be obtained by subtracting the colorimetric reading from 100 and multiplying by the factor 0.002 if the dilution was to 10 cc., by 0.003 if the dilution was to 15 cc., etc.

In case it is desired to employ the Duboscq colorimeter this may readily be adapted to the above procedure by using a picramic acid solution which is the exact equivalent of the color of 0.6 mg. of glucose treated as above and diluted to 10 cc.[9] When the standard is set at 10 mm., a blood containing 0.10 per cent sugar manipulated as above with 10 cc. dilution, will match at 10 mm., since the 3 cc. of the picric acid filtrate employed are the equivalent of 0.6 cc. of blood. For the calculation the following formula, in which D represents the dilution and R the colorimetric reading may be employed, $\frac{D \times 0.1}{R}$ = blood sugar in per cent.

[7] This is prepared by dissolving 0.100 gm. of picramic acid and 0.2 gm. of anhydrous sodium carbonate in 30 cc. of warm distilled water and finally diluting to 1 liter.

[8] The scale has been inverted and dropped 2 mm. in the instrument modified by Rowntree for the phenolsulphonephthalein test. This scale may be made to correspond with the regular instrument by subtracting the reading found from 100 and adding 2.

[9] Glucose in picric acid solution keeps permanently. 3 cc. of 0.2 per cent glucose solution in saturated picric acid, treated with 1 cc. of 20 per cent sodium carbonate and heated as above may serve to standardize the picramic acid or, in fact, may be used as the standard itself. Such a solution is likewise valuable for use as an occasional control on the method. Although the picramic acid standard is practically permanent, it is best not to give it undue exposure to strong light. For the Duboscq the standard needs to be about two-thirds as strong as for the Hellige.

When only an occasional blood sugar estimation is to be made, evaporation according to the original Lewis-Benedict method is not a great hardship. More than half the time required may be saved, however, by utilizing the 1 to 5 dilution employed above. Theoretically, the higher dilution is to be preferred, but practically we believe that with the lower dilution the extraction of the sugar is sufficiently complete to be within the limits of accuracy with the method. It may be argued that the color is not completely developed without evaporation. Should there be a slight difference (and it is by no means easy to demonstrate), it would not impair the accuracy with the Hellige instrument, since the curve is plotted according to this technique. With a 10 cc. final dilution each division on the scale (1 mm.) represents a difference of 2 per cent. If carefully carried out we believe the accuracy can be made to fall within 1 to 3 per cent. In most observations on the human subject, however, we do not consider that it is advantageous or desirable to compute results beyond the second decimal place. Differences which do not fall within these two figures can possess no practical significance. Utilizing the technique described, we have found it possible to make quantitative recoveries of glucose added to blood. Our early observations (made on hot solutions) led us to believe that the creatinine of uremic bloods would appreciably influence the accuracy of the sugar determinations. The color produced by the creatinine fades so rapidly on cooling and diluting, however, that there is very little, if any, influence on the result.

If for any reason the use of the autoclave is found advantageous or desirable, a 1:5 dilution of the blood is to be preferred, since a more suitable color is obtained for colorimetric reading.

Considerable experience with the method described above has convinced us that it may advantageously be employed for many other purposes aside from the estimation of blood sugar.

Observations upon Hyperglucemia with Special Reference to the Influence of Renal Disease.

Some rather interesting observations on hyperglucemia in both diabetes and nephritis have been obtained with the above method on various hospital cases. On account of their theoretical interest and the practical value they show the estimation of blood

sugar to have, these observations bear brief discussion. That hyperglucemia exists in severe nephritis has been recognized for some time,[10] although a satisfactory explanation has not been advanced. The influence of the nephritis which generally accompanies advanced diabetes upon the threshold point of sugar excretion is likewise a topic of considerable interest. When the hyperglucemia is lowered by dietary restrictions, the blood sugar in certain of these cases does not return to normal even with the disappearance of sugar from the urine. Without doubt in many of these cases the threshold point of sugar excretion has been raised by the nephritis, and furthermore it is probable that the nephritis exerts its usual elevating influence upon the sugar content of the blood.

Most of our determinations on cases with no known cause for hyperglucemia have shown values between 0.09 and 0.11 per cent, in confirmation of Lewis and Benedict. There are a considerable number of hospital cases, however, which show blood sugars of 0.12 to 0.14 per cent. The observations tabulated below represent the more interesting results obtained from about 500 analyses.[11] As a rule, the samples of blood have been obtained in the morning before breakfast.

In Table I are shown some rather pronounced hyperglucemias encountered in severe cases of nephritis. The first two cases in this table were fatal cases of mercuric bichloride poisoning. The next group of six cases suffered from chronic interstitial nephritis with uremia and were all fatal except the last. Glucosuria was not noted in the cases of mercury poisoning or of those suffering from interstitial nephritis. The last group of two cases suffered from chronic parenchymatous nephritis, and both showed hyperglucemia with an almost constant mild glucosuria.

Pavy[12] and other older investigators, and recently McLean,[13] have attempted to show that there is a direct relation between

[10] Compare Bang, I., Der Blutzucker, Wiesbaden, 1913, 128; also Neubauer, E., *Biochem. Z.*, 1910, xxv, 284; Rolly, F., and Oppermann, F., *ibid.*, 1913, xlviii, 268; Hopkins, A. H., *Am. Jour. Med. Sc.*, 1915, cxlix, 254.

[11] Most of these cases were patients in the medical wards of the hospital and we are indebted to Professor Edward Quintard and other members of the Medical Staff for the opportunity of studying these cases.

[12] Pavy, F. W., Physiology of the Carbohydrates, London, 1894, p. 193.

[13] McLean, F. C., *Jour. Am. Med. Assn.*, 1914, lxii, 917.

TABLE I.

Hyperglucemia in Nephritis.

Group.	Case.	Age.	Sex.	Date.	Sugar of blood.	Urea N of blood per 100 cc.	Remarks.
		yrs.			*per cent*	*mg.*	
I	W. F.	25	♂	Nov. 16, 1914	0.18	182	Fatal cases of poisoning with mercuric bichloride.
	—	29	♀	Oct. 27, 1915	0.17	65	
II	W. O'C.	33	♂	Mar. 7, 1914	0.19	200	
				" 9, 1914	0.20	182	
	M. K.	42	♂	May 13, 1914	0.18	140	
	I. D.	17	♀	Dec. 21, 1914	0.19	134	
				" 30, 1914	0.19	170	
	E. M.	39	♂	Nov. 30, 1915	0.18	97	
				Dec. 7, 1915	0.18	129	Cases of chronic interstitial nephritis with uremia, all fatal except the last.
				" 10, 1915	0.22	132	
	T. D.	34	♂	Jan. 22, 1915	0.09	60	
				Feb. 6, 1915	0.12	72	
				" 12, 1915	0.14	66	
				Mar. 9, 1915	0.14	97	
				" 16, 1915	0.14	120	
	J. W.	34	♂	Feb. 24, 1915	0.14	55	
				Mar. 1, 1915	0.10	63	
				" 6, 1915	0.10	89	
				Apr. 15, 1915	0.14	144	
	L. P.	57	♂	Jan. 6, 1915	0.14	80	
				Feb. 18, 1915	0.10	59	
				Mar. 1, 1915	0.10	37	
				" 5, 1915	0.10	45	
III	C. M.	50	♂	Apr. 15, 1915	0.20	15	
				" 27, 1915	0.19	16	Cases of chronic parenchymatous nephritis; generalized edema; high blood pressure; large amounts of albumin and many casts in urine; low phthalein outputs; almost constant mild glucosuria, generally under 1 per cent.
				Dec. 7, 1915	0.17	11	
	J. P.	62	♂	July 27, 1915	0.18	28	
				Aug. 31, 1915	0.18	23	
				Sept. 24, 1915	0.17	25	
				Oct. 1, 1915	0.14	25	
				" 26, 1915	0.14	29	
				Nov. 19, 1915	0.16	28	
				Dec. 3, 1915	0.18	25	

TABLE II.

Influence of Nephritis upon the Excretion of Sugar in Diabetes.

Case.	Age.	Sex.	Date.	Sugar of blood.	Sugar of urine.	Severity of nephritis.	Remarks bearing upon nephritis.
				per cent	*per cent*		
M. G.	63	♀	June 3, 1914	0.19	0	++	Phthalein output 30 per cent; blood pressure (systolic-diastolic) 185–100; small amount of albumin and few casts in urine; no edema.
F. R.	47	♂	Feb. 17, 1915	0.22	0	++	Blood pressure 190–100; trace of albumin and few hyaline and granular casts constantly present in urine.
C. L.	57	♀	" 18, 1915	0.24	5.0	++	Trace of albumin and hyaline and granular casts in urine; endarteritis obliterans; arteries markedly sclerosed.
E. S.	27	♀	May 15, 1915	0.31	6.0	+	Trace of albumin at times; few hyaline casts; polyuria; no edema, headaches; blood pressure 95–60.
M. McC.	61	♀	Apr. 15, 1915	0.33	0.5	++	Blood pressure 190–110; urine small in volume; many casts; large amount of albumin; endarteritis obliterans.
S. F.	61	♂	Nov. 20, 1915	0.33	6.3	+	Few hyaline casts; albumin from prostatitis.
A. M.	68	♂	" 6, 1915	0.34	0	+++	Large amount of albumin; many granular casts; blood pressure 160–90; died.

TABLE II—*Continued.*

Case.	Age.	Sex.	Date.	Sugar of blood.	Sugar of urine.	Severity of nephritis.	Remarks bearing upon nephritis.
				per cent	*per cent*		
O. U.	60	♂	Oct. 8, 1915	0.35	Trace.	?	*Diagnosis, carcinoma of head of pancreas;* symptoms of obstruction of common duct 6 wks.; no albuminuria.
J. E.	23	♀	June 4, 1914	0.36	7.0		Clinic case; no nephritic symptoms.
R. J.	47	♂	Mar. 16, 1915	0.36	1.2	+++	Phthalein 36 per cent; moderate albuminuria, granular casts; blood pressure 200–100; headaches and failing eyesight.
M. W.	53	♂	Apr. 10, 1915	0.37	1.7	++	Phthalein 44 per cent; albuminuria, blood picture of interstitial nephritis; only slight acetonuria; died.
C. R.	35	♀	Mar. 21, 1915	0.38	6.2	?	Diabetes 6 yrs.; amputation of left foot for gangrene.
P. F.	48	♀	June 4, 1914	0.39	2.2	++	Phthalein 42 per cent; moderate albuminuria; only slight acetonuria.
A. McD.	30	♂	Mar. 21, 1915	0.42	5.0	?	Occasional slight albuminuria; marked edema.
B. S.	46	♀	Aug. 26, 1915	0.42	3.6	+++	Excessive albuminuria; many granular casts; marked acidosis; died in coma.

TABLE II—*Concluded.*

Case.	Age.	Sex.	Date.	Sugar of blood.	Sugar of urine.	Severity of nephritis.	Remarks bearing upon nephritis.
				per cent	*per cent*		
J. C.	50	♀	Nov. 26, 1915	0.46	0	+++	Phthalein 34 per cent; occasional trace of albumin; few casts; blood pressure 190–130; diabetes 15 yrs.; sugar excretion 0–2.5 per cent.
M. McC.	56	♀	Feb. 12, 1915	0.57	8.0	+	Phthalein 52 per cent; trace of albumin in urine on admission; no edema; blood pressure 176–105; carbohydrate tolerance 80 gm.
R. B.	15	♂	Mar. 10, 1914	0.79	8.7	+	Marked polyuria; considerable acidosis; occasional trace of albumin in urine; tuberculosis.
L. F.	52	♀	Oct. 29, 1915	0.80	2.2	++	Patient died day after observation; albuminuria; nitrogen retention; see first case of next table.

the degree of hyperglucemia and glucosuria, a view well supported by their observations.

Table II gives the concentration of the sugar in both the blood and urine in nineteen severe cases of diabetes. Most of the observations were obtained before the patients had been placed upon a greatly restricted diet; *i.e.*, at a time most favorable to the development of hyperglucemia and glucosuria. An inspection of the table shows that most of these patients likewise suffered from nephritis of more or less severity. Although some cases

with definite nephritic symptoms retained the power of secreting a urine of high sugar content, severe nephritis appears to reduce markedly the permeability of the kidney for sugar; and it is only when these latter cases are excluded from the above table that there appears to be any relation between the hyperglucemia and glucosuria. Most writers on diabetes mention the influence of nephritis upon the permeability of the kidney for sugar. Although the above table probably gives an exaggerated impression of the occurrence of nephritis in diabetes, since the cases were those needing hospital care, it is not believed that the importance of this subject has hitherto been fully appreciated.[14] It is worthy of note that a number of the above cases with reduced carbohydrate intakes, but with blood sugars above 0.2 per cent, showed a disappearance of sugar from the urine. When the threshold point of sugar excretion has been raised by a nephritis, the disappearance of sugar from the urine or a low sugar excretion is a poor guide to the glucemia. This would appear to be a factor of considerable importance in the application of Allen's starvation treatment in diabetes. In their most recent communications, both Allen[15] and Joslin[16] refer to the estimation of blood sugar, although they have not as yet drawn any conclusions as to its value in this connection.

The data on three of the fatal cases of diabetes deserve more detailed consideration. As shown in Table III, the blood sugar content of the first two of these cases was very high, the second determination on L. F. being slightly higher than any we have found recorded, although several cases mentioned by von Noorden[17] are very close to this figure, 1.10 per cent. In one case he noted a blood sugar of 1.01 per cent and emphasized that nephritis was not present. Lépine[18] likewise reports a case with a blood sugar of 1.06 per cent. In discussing the influence of nephritis upon the permeability of the kidney for sugar, von

[14] Compare Liefmann, E., and Stern, R., *Biochem. Z.*, 1906, i, 299; also Rolly, F., and Oppermann, F., *ibid.*, 1913, xlix, 278.

[15] Allen, F. M., *Am. Jour. Med. Sc.*, 1915, cl, 480.

[16] Joslin, E. P., *Am. Jour. Med. Sc.*, 1915, cl, 485.

[17] von Noorden, C., Metabolism and Practical Medicine, English edition by Hall, London, 1907, iii, 532.

[18] Lépine, R., La Diabète Sucré, Paris, 1909, 454.

Noorden writes: "I have myself drawn attention to an observation on a diabetic patient who died from uremic coma (not diabetic coma). The urine contained 1.4 per cent of sugar and the blood at the same time, 0.85 per cent."

The chemical blood pictures of the cases in Table III are typical of interstitial nephritis;[19] *viz.*, high uric acid values with more

TABLE III.

Composition of Blood in Three Fatal Cases of Diabetes.

Case.	Age.	Sex.	Date.	Sugar of blood.	Sugar of urine.	Uric acid.	Urea N.	Creatinine.	CO_2 — combining power (Van Slyke). Per 100 cc. of plasma.
						Per 100 cc. of blood.			
			1915	*per cent*	*per cent*	*mg.*	*mg.*	*mg.*	*mg.*
L. F.*	52	♀	Oct. 29	0.80	2.2	10.5	55	2.1	72
			" 30	1.10	0.5				61
M. W.**	53	♂	Apr. 10	0.37	1.7	6.0	18	2.0	
			" 17	0.98	1.6				
B. S.†	46	♀	Aug. 26	0.42	3.6	7.6	28	4.7	21

* Duration of disease, several years; no coma until day of death on Oct. 30. Small amount of albumin in urine of Oct. 26; 2.7 per cent of sugar; only faint trace of acetone in urine.

** Gangrene of toes at times during the last 5 yrs.; glucosuria very severe early in disease but later improved; systolic blood pressure 150–165; phthalein output 44 per cent; moderate amount of albumin in urine of Apr. 10; at death (Apr. 18) urine showed only small amount of acetone; uremic symptoms 30 hrs. before death and about 6 hrs. previous to the last blood analysis.

† Patient entered hospital in coma and died several hours later; urine contained very large amounts of albumin, acetone, and diacetic acid, and many granular casts.

or less increase in the urea. In the first two cases the creatinine was normal, although in the last case there was marked retention. The cause of death in the first two cases is obscure. It would scarcely appear to have been due to acidosis. The last case, however, showed marked acidosis, as ascertained from both the

[19] Compare Myers, V. C., Fine, M. S., and Lough, W. G., *Proc. Soc. Exper. Biol. and Med.*, 1915, xiii, 5; see also von Noorden, *loc. cit.*, 669.

urine and the CO_2-combining power of the blood (Van Slyke). In this case the marked nephritic symptoms, coupled with the high creatinine, would seem to indicate that the nephritis had as much to do with the cause of death as the diabetes.

TABLE IV.

A Comparison between Diabetes Mellitus and Renal Diabetes.

Case.	Age.	Sex.	Date.	Sugar of blood.	Sugar of urine.	Diet.	Remarks.
			1915	*per cent*	*per cent*		
			May 15	0.31	6.0	50 gm. carbohydrate.	Severe diabetes; much emaciated; no edema; nervous symptoms; mild nephritis as evidenced by occasional trace of albumin in urine and few hyaline casts.
			" 17	0.30	1.5	Hunger and whiskey.	
E. S.	27	♀	" 19	0.32	1.5		
			" 25	0.28	0.2		
			June 1	0.17	0		
			" 8	0.27	0.6	15 gm. carbohydrate.	
			Aug. 3	0.09	1.0	Hunger.	Probable "renal diabetes;" constant glucosuria for 7 yrs. in spite of treatment; at time of examination 4 mos. pregnant; no edema; greatly emaciated; thyroid slightly enlarged; blood pressure 95–60; trace of albumin and few finely granular casts in urine; phthalein 39 and 42 per cent.
			" 5	0.11	1.6		
			" 11	0.10	3.1	Green vegetables.	
			" 25	0.09	1.3	15 gm. carbohydrate.	
M. B.	31	♀					

The concentration of blood sugar is probably more important in the recognition of non-diabetic conditions than diabetes proper. In Table IV are given observations on a case of "renal diabetes" and for comparison somewhat similar observations on a case of diabetes mellitus. With the latter considerable difficulty was encountered in obtaining a disappearance of sugar from the urine,

even with complete starvation (and here hyperglucemia was still present). The case appears similar to one recently reported by Joslin, although probably not as severe. The blood sugar values in M. B. were essentially normal. Neither the sugar of the blood or urine was especially influenced by dietary restrictions. Although "renal diabetes" is not an uncommon condition in pregnancy,[20] the history of the case stamps it as true "renal diabetes." At about the same time our attention was directed to a number of other pregnant women who excreted very small amounts of sugar. Application of the phenylhydrazine test, according to Cole,[21] showed the sugar to be lactose in every case.

DISCUSSION AND SUMMARY.

The Lewis-Benedict method for the estimation of blood sugar may be greatly simplified by employing an initial blood dilution of 1:5 instead of 1:12.5. In this way a sufficiently low concentration is obtained to obviate the necessity for evaporation in the development of the color. For this determination the Hellige colorimeter has been found especially satisfactory.

A number of cases of nephritis are reported with hyperglucemias close to 0.2 per cent. In the four cases of interstitial nephritis glucosuria was absent, while mild glucosuria was constantly present in the two cases of parenchymatous nephritis with edema. The blood sugar content of nineteen advanced cases of diabetes is reported together with the corresponding sugar excretion. Many of the cases gave evidence of nephritis, apparently of the interstitial type. If sugar was being excreted by these cases, the percentage output compared to the blood sugar was correspondingly lower than in other cases of diabetes. As recently emphasized by Mosenthal,[22] cases of interstitial nephritis secrete a urine of a very constant low specific gravity with low content of chloride and nitrogen. It is possible that this same factor may have some influence on the concentration of urinary sugar. A case is reported with 1.10 per cent of sugar in the blood and only

[20] Novak, J., Porges, O., and Strisower, R., *Z. f. klin. Med.*, 1913, lxxviii, 413.

[21] Cole, S. W., *Lancet*, 1913, clxxxv, 859.

[22] Mosenthal, H. O., *Arch. Int. Med.*, 1915, xvi, 733.

0.5 per cent in the urine. If the nephritis is of the interstitial type, the data obtained for uncomplicated nephritis explain the raising of the threshold point of sugar excretion in these advanced cases of diabetes. The nephritis may further explain the difficulty in reducing the blood sugar of these cases to normal by restrictions in the carbohydrate intake. The use of lactose as a functional kidney test has shown how quickly the permeability of the kidney is impaired for this sugar in nephritis. As an index of the ability of the kidney to excrete sugar, it seems possible that a ratio between the sugar of the blood and urine might be worked out somewhat after the method McLean[23] has recently employed for urea and chlorides.

Figures for the sugar of the blood and urine are also reported in a case of "renal diabetes."

[23] McLean, F. C., *Jour. Exper. Med.*, 1915, xxii, 212 and 366. Compare Ambard, L., Physiologie normale et pathologique des reins, Paris, 1914, 74.

IS AUTOLYSIS AN AUTOCATALYTIC PHENOMENON?

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(Received for publication, December 8, 1915.)

As far as the writer is aware, no attempt has been made to determine the hydrogen or hydroxyl ion concentration of autolyzing digests. In an analytic study of the action of the enzymes involved, it is important to know the range of concentration of these components; for until the present time it has not been known what induces autolytic reactions, what changes the ionic concentration from a preponderance of hydroxyl ions to hydrogen ions, whether the hydrogen ion acts as a co-enzyme, activating the pro-enzyme of the tissues, or whether the process can be placed in the category of Ostwald's "autocatalysis,"[1] in which the process of enzyme action is accelerated by the products formed during the action of the enzyme and in consequence of it. From the pathological standpoint it is desirable to know the range of acidity developed in an atrophying tissue.

Wiener,[2] and Baer and Loeb[3] have studied the reaction of the medium in autolyzing digests, and the two reports are similar in respect to determinations of the range of acidity in which the enzymes of autolysis are active. Wiener finds that "bei einer Konzentration, die 0.2–0.4 per cent NaOH entsprach, die *Autolyse vollständig aufgehoben wird;*"[4] and that "*bei einer Alkaleszenz, wie sie der Blutalkaleszenz entspricht* und auch bei einer doppelt so hohen, *eine Autolyse* wenigstens durch eine Wochelang *ausgeschlossen ist.*"

Since the time of Hoppe-Seyler,[5] it has been customary to attribute the inception of autolysis to the development of products of intermediate oxidation, as the oxygen supply is reduced in cases involving autolysis *in vivo;* such compounds as lactic acid, etc., are known to be formed, and it is conceivable that the appearance of these compounds marks the beginning of autolysis. However, the writer has studied the rôle of oxygen in autolyzing tissue[6] with the result that no apparent effect seems to be produced

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[1] Ostwald, W., *Lehrb. d. allg. Chem.*, 1910, ii, pt. ii, Verwandtschaftsl., 263.

[2] Wiener, H., *Zentr. f. Physiol.*, 1905–06, xix, 349.

[3] Baer, J., and Loeb, A., *Arch. f. exper. Path. u. Pharm.*, 1905, liii, 1.

[4] Wiener, *loc. cit.*, 355.

[5] Hoppe-Seyler, F., *Med.-Chem. Untersuch.*, 1877, iv, 499.

[6] Morse, M., *Biochem. Bull.*, 1915, December (in press).

upon the rate of autolysis when oxygen gas or oxygen-liberating compounds are introduced into the digests. The fact that autolysis apparently proceeds in muscles where the blood system is intact, but the nerve supply interrupted,[7] indicates that the assumption which has been made concerning oxygen as the factor determining autolysis should be investigated. The rôle of starvation in inducing autolytic changes has been studied by some workers (Rettger,[8] for bacteria, etc.), where reduction in food induces autolytic processes. That autolytic enzymes are specific in some cases (Jacoby[9]) and heterolytic[9] in others has been the experience of other workers (Bradley[10]), so that it is difficult to explain the rise of autolytic changes in a given tissue in terms of the throwing off of the effects of any anti-enzyme, such, for instance, as Jobling[11] and his coworkers have found for trypsin in the unsaturated fatty acids and their soaps. In all the possible explanations of inception of autolysis, the only factor which seems common is the alteration in hydrogen ion concentration.

Bradley[10] has determined that an alteration probably occurs in the tissue substrate through the action of certain introduced reagents and perhaps through the action, likewise, of the acid normally produced during the process of autolysis. From the results presented here, it is evident that a relatively low hydrogen ion concentration is developed at the inception of the process and that the increase as autolysis proceeds is small and runs *pari passu* with the curve of soluble nitrogen. If the effect of acid production during autolysis were, as Bradley holds, of "two effects: (1) it will remove the inhibitory alkalinity, and (2) alter the proteins with a resulting increase in the mass of substratum,"[12] then we should imagine that the curve of the rate of autolysis in a normally digesting hash would show a latency at the beginning while the acid produced is being absorbed by the proteins, so that no free acidity is available for the reactions. Wiener[2] and Magnus-Levy[13] have recorded such a latency, but the present writer has not observed it in any digests, although the tissue was submitted to Salkowskian autolysis immediately after removal from the body. All curves of autolysis rate show[14] that the rise of curve is abrupt from the beginning, the greatest digestion proceeding within the first 12 hours of action. The only alternative to this conclusion is that the acid produced is so great that the tissue becomes saturated, with excess of acid. In this case, we should expect to have a sudden rise in acidity at a time when saturation of the proteins has been completed, representing the acid no

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[7] Morse, *Am. Jour. Physiol.*, 1915, xxxvi, 147.

[8] Rettger, L. F., *Jour. Med. Research*, 1904–05, xiii, 79.

[9] Jacoby, M., *Beitr. z. chem. Phys. u. Path.*, 1903, iii, 446.

[10] Bradley, H. C., *Jour. Biol. Chem.*, 1915, xxii, 113.

[11] Jobling, J. W., and Petersen, W., *Jour. Exper. Med.*, 1914, xix, 239 ff.

[12] Bradley, *loc. cit.*, 123.

[13] Magnus-Levy, A., *Beitr. z. chem. Phys. u. Path.*, 1902, ii, 261.

[14] Compare Bradley and Morse, *Jour. Biol. Chem.*, 1915, xxi, 212 ff. Morse, *Jour. Biol. Chem.*, 1915, xxii, 125.

longer taken up by the proteins and thus remaining free for participation in the reaction. While such a condition is possible, we should seek the simpler explanation, that the acidity developed acts catalytically, activating the enzyme in much the same manner that the hydrochloric acid of gastric digestion activates; for there is nothing to indicate a sudden alteration in the $H^{\cdot}$ content in the curves. The extent of digestion in an autolyzing hash does not require the assumption that the substrate has been affected so that more is present and capable of being digested; if the globulin portion is digestible, as Jacoby[15] determined and as the present writer has also found, the figures of Pohl[16] for liver globulin fraction are adequate to account for the digestion in autolysis without the assumption of increase of substrate. If any modification were to occur in the acid, it would be logical to assume that the conversion would tend towards globulin-like bodies, but the only case where a change of this nature has been reported has involved the action of alkali[17] and not of acids.

The method followed in the determination of the hydrogen ion concentration is briefly as follows: Thymus was used as substrate, owing to the colorless extract. Liver, which has been used in the writer's previous work on autolysis, cannot be used to advantage, the colorimetric determinations being rendered inexact by the retention of the reddish colors of the extracts. The method of preparation of the substrate was similar to that given in previous papers by the present writer. The preparation of the standard color solutions followed the method given by Sörensen[18] and in part the descriptions of Levy, Rowntree, and Marriott.[19] The following solutions were used:

Salt M/15.	Proportions in cc.													
	Methyl orange.						Phenolsulphonephthalein.							
P_H	4.5	4.9	5.3	5.6	5.9	6.2	6.4	6.6	6.8	7.0	7.1	7.2	7.3	7.4	7.5
KH_2PO_4	10.0	9.9	9.75	9.5	9.0	8.0	7.3	6.3	5.1	3.7	3.2	2.7	2.3	1.9	1.58
Na_2HPO_4	0.0	0.1	0.25	0.5	1.0	2.0	2.7	3.7	4.9	6.3	6.8	7.3	7.7	8.1	8.42

The digests were removed for sampling and 10 cc. of the liquid were removed after shaking. The withdrawn liquid was placed in Schleicher and Schüll protein-impermeable dialyzing tubes.

[15] Jacoby, M., *Ergebn. d. Physiol.*, 1902, i, 213.

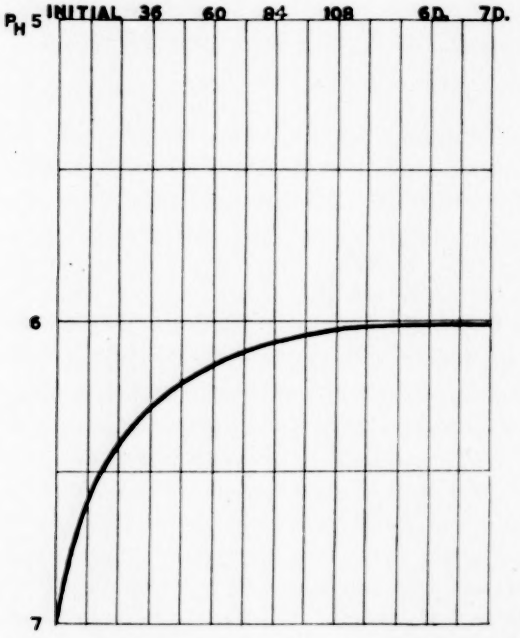
[16] Pohl, J., *Beitr. z. chem. Phys. u. Path.*, 1906, vii, 381.

[17] Moll, L., *Beitr. z. chem. Phys. u. Path.*, 1904, iv, 562.

[18] Sörensen, S. P. L., *Ergebn. d. Physiol.*, 1912, xii, 393.

[19] Levy, R. L., Rowntree, L. G., and Marriott, W. McK., *Arch. Int. Med.*, 1915, xvi, 389.

which had previously been tested to insure that they were protein-impermeable, and the tubes were then placed in 50 cc. of distilled water. The control distilled water gave no color reaction with the indicators to warrant the preparation of conductivity water. The dialysis proceeded exactly 5 minutes, when the dialysate was removed to the extent of 3 cc., the amount in the standards. One drop of indicator was added, the preparation shaken thoroughly, and the colors were matched. Tubes of uniform size were used throughout (Wassermann tubes). The results are plotted in terms of the Sörensen nomenclature, P_H = log. conc. $H^{\cdot}$. Determinations were made at 12 hour intervals for the first 48 hours and thereafter at 24 hour intervals, all experiments being made in duplicate.



This curve is to be compared to the curves of autolysis rate in normal autolyzing hash.[20] The curves are similar in essentials. In both instances we find an abrupt rise, the greatest digestion, and the greatest development of acid occurring within the first 60 hours. As the curve of acidity approaches the plateau, the curve of digestion likewise reaches the maximum.[21]

[20] Compare references given above.

[21] There was no appreciable change in acidity after the fifth day.

It may be considered a debatable point whether the digestion is responsible for the similarity in the curves or whether it is a question of the effect of the acid. When we remember that the development of acidity is the *sine qua non* for autolysis, we have reason to believe that the process is an autocatalytic one, the developing acidity inducing greater and greater acceleration in the digestion rate, and when the acid reaches a maximum in its production, digestion likewise reaches its maximum.[22]

CONCLUSIONS.

1. Autolysis is an autocatalytic phenomenon, the products of digestion entering into the reaction as true catalyzers.
2. There is nothing in the study of acid production to indicate that the substrate is altered by the acid.
3. The present report substantiates studies of other investigators in regard to the degree of acidity necessary in autolysis: *e.g.*, Wiener, and Baer and Loeb; but no determination of acidity developed during digestion seems to have been made previously.

[22] Titrations with 0.05 N $Ba(OH)_2$, with thymolphthalein as indicator, yield results similar to those given by the colorimetric method. In this case protein-free filtrates were used. The curve is essentially similar to the one presented, which may be taken as the type of all curves concerned with the reaction of the medium in autolyzing digests.

THE OCCURRENCE OF *p*-HYDROXYPHENYLETHYLAMINE IN VARIOUS MISTLETOES.

By ALBERT C. CRAWFORD and WALTER K. WATANABE.

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In 1914 we reported[1] that *Phoradendron flavescens*, the mistletoe of our southeastern states, owed most, if not all of its pressor activity to *p*-hydroxyphenylethylamine. This raised the question whether this compound occurred in other species of mistletoe. In some of the work reported no distinction in varieties has been made, and this may lead to confusion. The classification of the genus *Phoradendron* is now under discussion.[2]

European workers have reported that *Viscum album*, the European mistletoe, will on intravenous injection into dogs cause a persistent fall in blood pressure with increase in urinary secretion. According to Selig, extracts of *Viscum album* increase the amount of urine secreted during the 24 hours. Extracts of this species of mistletoe have been introduced into therapeutics as a hypotensive agent.[3]

According to Chevalier,[4] *Viscum album* contains a volatile alkaloid, a resinous substance with a drastic action, and two glucosides. To these latter Chevalier ascribed the hypotensor and diuretic action. The volatile alkaloid was claimed to cause a transitory rise in blood pressure, stimulation of the medulla, and salivary and bronchial hypersecretion.

Barbieri[5] claims that the activity of *Viscum album* is not due to a saponin. While there has been much discussion as to the active compound in this plant, no one has yet isolated the hypotensor agent. Leprince[6] has obtained from this plant a volatile base having the composition $C_8H_{11}N$, but

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[1] Crawford, A. C., and Watanabe, W. K., *Jour. Biol. Chem.*, 1914, xix, 303.

[2] Trelease, W., *Proc. Nat. Acad. Sc.*, 1915, i, 30.

[3] Selig, A., *Med. Klin.*, 1912, viii, 991.

[4] Chevalier, J., *Compt. rend. Soc. de biol.*, 1908, lxiv, 2. Lesieur, E., Contribution à l'étude du Gui, Thèse de Paris, 1910.

[5] Barbieri, O., *Arch. di farm. sper.*, 1913, xiv, 39.

[6] Leprince, M., *Compt. rend. Acad.*, 1907, cxlv, 940.

there is no report as to whether the volatile compound of Leprince is the same as that referred to by Chevalier.

We made several attempts to secure samples of the European mistletoe, and finally one of the Eastern drug firms sent us a fluid extract which they stated was made from *Viscum album*. This fluid extract caused a rise in blood pressure like that produced from *Phoradendron flavescens*, and we suspected that the firm was confusing the genus and using the American plant. Ostenberg[7] found *p*-hydroxyphenylethylamine in this fluid extract, and, trusting to the firm's statement, reported that *Viscum album* contained this compound.

Since the appearance of Ostenberg's paper, we have obtained *Viscum album* from Italy and also from England.[8] Fresh 30 per cent alcoholic extracts of both lots caused a fall in blood pressure in dogs with cut vagi, and from these extracts we obtained no *p*-hydroxyphenylethylamine by the methods described in our previous papers. The ether extract of the alkaline solution yielded an oxalate, but the intravenous injection of 15 mg. did not cause a rise in blood pressure.

A specimen of *Phoradendron villosum*, which was parasitic to *Quercus lobata*, was obtained from the Stanford campus. This sample contained leaves and twigs. A 30 per cent alcoholic extract of this plant produced a rise in blood pressure and we obtained an oxalate, by our method, which raised the blood pressure and accelerated the cardiac rate in dogs. From the oxalate a dibenzoyl compound was made which melted at 170°C., corresponding to the melting point of the dibenzoyl compound of *p*-hydroxyphenylethylamine.

Phoradendron villosum parasitic to an oak (variety not given) was obtained near Redwood City, California. A 30 per cent alcoholic extract of this produced a rise in blood pressure and yielded an active oxalate.

Arceuthobium occidentale (Jepson), parasitic to a Monterey pine (*Pinus radiata*), was obtained from near Pacific Grove, California. An alcohol extract (1 cc. = 1 gm. of the fresh plant) produced no rise in blood pressure. This plant yielded no oxalate.[9]

[7] Ostenberg, Z., *Proc. Soc. Exper. Biol. and Med.*, 1915, xii, 174.

[8] The plants from England were given us by Parke, Davis and Co., and the identification was confirmed through the courtesy of Dr. W. W. Stockberger of the U. S. Dept. of Agriculture. Those from Italy were obtained through Professor R. Dohrn of Naples, Italy.

[9] This plant was obtained through the kindness of Professor George Peirce.

Professor Thornber, of the University of Arizona, sent us samples of *Phoradendron flavescens*, variety *macrophylla* and *Phoradendron californicum*. Intravenous injections of an alcohol-aqueous extract of both plants caused a rise in blood pressure similar to that produced by *Phoradendron flavescens* and from both were obtained a dibenzoyl compound, a picrate, and an oxalate, which had the same melting points as those made from the base we obtained from *Phoradendron flavescens*. Extracts of a sample of *Phoradendron villosum* obtained from Professor Thornber produced no rise in blood pressure and yielded no *p*-hydroxyphenylethylamine, although it yielded an inactive oxalate from an ethereal shaking of an alkaline extract. This sample contained no leaves.[10] Whether this difference in the results obtained from the Arizona and the California varieties is due to the fact that in one case the sample contained leaves and in the other none, has not been investigated.

30 per cent alcoholic extracts of *Phoradendron bolleanum*,[11] *Phoradendron juniperinum*, and *Razoumofskya cryptopoda* (Coville) in which 1 cc. was supposed to represent 1 gm. of the dried plants, were injected intravenously into dogs. It was found that the extract of *Phoradendron bolleanum* (1.5 cc.), in one experiment, produced a slight rise in blood pressure, but in another experiment 1 cc. produced merely a fall. From this plant a small amount of an oxalate was obtained, but not sufficient to make a blood pressure test. It reacted with Moerner's test like *p*-hydroxyphenylethylamine. The extract of *Razoumofskya cryptopoda* and of *Phoradendron juniperinum* produced a fall in blood pressure, but no rise. Neither yielded an oxalate.

In 1911 one of us[12] reported that *Phoradendron juniperinum*, which was obtained from Arizona, seemed to be active, while *Phoradendron villosum*, obtained from the same state, seemed to be inactive. Unfortunately our

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[10] Extracts of both the pituitary and of the suprarenal glands yield an inactive oxalate from an ethereal shaking of an alkaline extract.

[11] These were received from Professor Thornber. The *Phoradendron bolleanum* was parasitic to the white fir, *Abies concolor;* the *Phoradendron juniperinum* grew on *Juniperus monosperna* and was obtained at an altitude of about 5,000 feet. *Razoumofskya cryptopoda* was parasitic to the Arizona pine, *Pinus arizonica*, in the high mountains of Arizona.

[12] Crawford, A. C., *Jour. Am. Med. Assn.*, 1911, lvii, 865.